

ANNUAL REVIEW OF MICROBIOLOGY

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VOLUME 10

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1956

ANNUAL REVIEWS, INC.
PALO ALTO, CALIFORNIA, U.S.A.

ANNUAL REVIEWS, INC.
PALO ALTO, CALIFORNIA, U.S.A.

FOREIGN AGENCIES

Maruzen Company, Limited
6, Tori-Nichome Nihonbashi
Tokyo

PRINTED AND BOUND IN THE UNITED STATES OF AMERICA BY
GEORGE BANTA COMPANY, INC.

Can. Rev. 1936

Publication

12 Oct 1936

12 Oct 1952
Editorial
Publisher

PREFACE

With great pleasure we express once again our gratitude to those authors who have contributed to the *Annual Review of Microbiology*. We have been fortunate in obtaining their cooperation in the preparation of timely and useful reviews in various fields of Microbiology. We regret that factors beyond the control of the authors and the publisher made it impossible to present reviews on Genetics of Bacteria and Viruses, by A. H. Doermann, Nematodes, by G. Steiner, Variations in Some Parasitic Flagellates, by S. Inoki, Neurotropic Viruses, by S. Gard, and Anti-Fungal Antibiotics, by F. Howard, A. Prince, and B. Champlin.

It is the hope of the Editorial Committee, and of the National Science Foundation, that the review of the Russian literature in general microbiology will prove of value to our readers.

At this time we wish to express our thanks to Dr. J. M. Sherman for his valuable contributions to this Review during the first ten years of its life. We are pleased to welcome Dr. Wayne Umbreit as Dr. Sherman's successor on the Editorial Committee.

We also express our appreciation for the valuable assistance rendered by the office staff of Annual Reviews, Incorporated, acknowledging in particular the aid given us by Mrs. Delores Ward as editorial assistant.

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FACTORS AFFECTING RESISTANCE TO INFECTION^{1,2}

BY SANFORD S. ELBERG

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Patterns of investigation on the subject of nonspecific resistance in the year under review have been greatly clarified by papers on organizational aspects as well as by specific contributions of a research nature. Miles, in two stimulating essays (1, 2) has analyzed the subject of bacterial invasion in terms of the constitutive and adaptive mechanisms with which the host is endowed. This method of analysis allows many hitherto unrelated yet nonetheless important observations to be considered as determinants or mediators of infection or resistance. The idea of determinants has been plausibly discussed by Dubos who suggests that the interplay between host and parasite within the microenvironment of inflammation should be the object of more study for a better understanding of the mechanisms by which latent infections are activated. The phagocytic theory and the survival value of the adaptive responses of cellular and humoral components of inflammation are re-examined by both Miles (1) and Dubos (3, 4, 5) not as to the oft-discussed significance of Metschnikow's observations on *Daphnia* but in terms of the chemical and enzymatic contribution of the inflammatory cells to the persistence and viability of the parasite.

CONSTITUTIVE PATTERNS

Genetic and physiological pattern.—Three reviews on this aspect of resistance in plants and animals have appeared (6, 7, 8). Russell (9) has examined the various categories of strain differences among animals in terms of the degree of fixation of the genes carried by a strain. The limitation imposed on the use of highly inbred colonies is such that for brother-sister matings to continue indefinitely, early abnormalities cannot be found in all or in a high proportion of individuals since this would interfere with strain continuation. The consequences of strain differences in inbred animals are such as to affect the nature of the disease or variation in tissue tropism of the parasite, alteration of survival time and correlative tolerance to parasitemia, hormonal content of the animal and its sensitivity to the hormone, as well as the type of reaction to the hormone by different tissues. Among the physiological patterns which appear in inbred strains are the red cell counts, hematocrit values, hemoglobin concentration, number and kind of leukocytes and the aging process. Ram & Hutt (10) have shown that the accelerated defense against

¹ The survey of literature pertaining to this review was completed in December, 1955.

² The following abbreviations have been used in this chapter: ACTH (adrenocorticotropin), and RES (reticulo-endothelial system).

infection by *Salmonella pullorum* is a strain characteristic wherein chicks from resistant families are characterized by a higher mean body temperature, and a superior thermoregulatory system during the first five days of age. Contrary to the findings of others, lymphocyte differences in the low- and high-temperature families were not significantly different and were unrelated to mortality from the infection. Upton *et al.* (11, 12), using the intracerebral route of infection in mice with Newcastle disease virus demonstrated a greater susceptibility and longer incubation period in three-day-old mice than in weanlings and adults. Nine-day-old mice were invariably more resistant to infection than three-day-old mice and more resistant than weanlings to the toxic effects of the virus. On the other hand, weanling and adult mice were more susceptible to the toxic effect of the virus than to infection. In the case of Histoplasmosis in mice, resistance increased more rapidly with age in female Swiss mice than in males (13). The effect of age on the resistance of rabbits to meningococcal toxin and to the development of the generalized Schwartzman reaction appears to be quite separate (14). Rabbits four to seven weeks old tolerated single doses of the toxin which in ten to twelve-week-old animals were quite lethal. On the other hand, the younger rabbits were more susceptible to the induction of the generalized Schwartzman reaction by two doses of toxin 24 hr. apart.

On the genetic side Haldane (8) has discussed certain biochemical abnormalities, and cited Takahara's series of nine cases of oral gangrene apparently attributable to acatalasaemia in three sibships all from marriages of normal cousins (15), suggesting that normal blood catalase serves as part of the defense against hemolytic streptococcus infections. Nadel *et al.* (16) have continued their earlier studies on the genetics of resistance of mice to malaria. Two thousand mice from 12 inbred strains and from 13 different F_1 hybrid crosses of these strains as well as backcrosses and F_2 hybrids from one of the more resistant hybrids were employed. It was significant that survival time was the critical index, since total parasitemia itself gave no evidence of strain differences in resistance. The evidence implicating genetic factors included heterosis, greater variance in the F_2 and in the backcross animals and significantly increased survival of the backcross to the long-survival parent. Extra-genetic factors were indicated by a wide range in survival in all series and a bimodal death curve. Examining the experimental animal in a most exhaustive fashion, Lurie, Zappasodi & Tickner (17, 18) have continued their elegant analysis of the nature of resistance to tuberculosis in inbred families of rabbits. Utilizing chronicity, localization, generalization, and death, as well as determinations of the numbers of bacilli required to produce a single tubercle, the authors have incidentally confirmed the conclusions of other workers dealing with different infectious agents; namely, that a more sensitive appraisal of host resistance is possible when organisms of less than maximum virulence are used. The difference in reactivity between susceptible and resistant families was of the same nature as the difference between animals of the same native resistance, one under the influence of

hydrocortisone, the other not. The inhalatory route of infection of rabbits by human type *Mycobacterium tuberculosis* proved to be the most sensitive test for resistance. The greater number of human-type bacilli required to generate a tubercle as contrasted to the "one bacillus-one tubercle" situation for the bovine strain allowed Lurie *et al.* (17, 18), in studying cortisone enhancement of infection in genetically resistant and susceptible rabbits, to conclude that the tuberculogenic capacity in both races of rabbits was not a primary function of virulence of the strain. The data presented by Lurie *et al.* clearly indicate that nonspecific constitutional factors affect the initial multiplication of bacilli, preventing tubercle formation, and one is faced with the conclusion that tubercle bacilli do persist in tissues without multiplication. More striking was the picture of dispersal of the infection as an attribute of the quality of resistance. Dissemination via lymphogenous and hematogenous routes was greater in the susceptible type rabbits, but the reason for this appeared to reside in the ability of the resistant rabbits not to localize the infection but to destroy the focalized bacilli in whatever area they invaded. Although lymph node invasion occurred rapidly in both families of rabbits, the rate of dispersion from the lung to the tracheobronchial nodes was actually greater in the resistant type rabbits at all intervals studied.

At the cellular level, resistance to intracellular growth of tubercle bacilli is under the influence of the hormone balance (18). Furthermore, Kass and co-authors (19) have observed that susceptible families of rabbits produce about twice the amount of 17-hydroxycorticosterone as do the resistant families. Infected susceptible families, however, do not increase the production of adrenal steroids over that produced by uninfected controls whereas infected resistant-type rabbits do increase their production of both 17-hydroxycorticosterone and corticosterone. Now, administration of ACTH induced the susceptible type animals to produce more of the 17-hydroxycorticosterone than was produced by uninfected susceptibles or infected susceptible animals not on ACTH. If the animals were infected, however, then the response to ACTH was to produce more corticosterone and relatively less 17-hydroxycorticosterone. This would further suggest that the qualities of resistance and susceptibility in tuberculosis are closely linked to the hormone balance and that tuberculosis itself may alter the responses of the adrenal cortex.

DETERMINANT PATTERNS OF RESISTANCE

Nutritional and metabolic.—The phases of resistance of animals to tuberculosis which relate to tissue metabolism and are thereby reversible have been investigated by Dubos (20) through modification of tissue chemistry by producing biochemical lesions and by *in vitro* study of the effects of analogous conditions and substances on the multiplication of the bacilli. Among such conditions were decreased glycogen stores, disruption of glycolytic activity, and increase in the content of acetone bodies and polycarboxylic acids in the body fluids. On the nutritional side, survival of infected mice was reduced

by a 30-hour fasting period in each of three successive weeks despite an adequate diet. Although a protein deficiency alone was not important in reducing resistance, it predisposed to increased susceptibility when the weight difference in the dietary composition was made up with cerelose, sodium citrate, or cocoa butter. On the other hand, when peanut oil was used to fortify the protein deficient diet no enhancement of infection was induced. In fact, peanut oil reversed the enhancing effect of cerelose on the protein deficient diet. In general sodium citrate enhanced susceptibility on a variety of diets and was not conditioned in its effect by a protein deficiency. Dinitrophenol and thyroid extract reduced survival time, especially in mice previously vaccinated with Bacille Calmette Guérin vaccine.

Hedgecock (21) reported that fatty acid esters used in the same proportions found in coconut oil prolonged survival time in mice to tuberculosis on a diet containing 20 per cent protein in the ration, but more or less protein decreased survival time. Replacement of the fatty acid mixture with 5 per cent lard or 0.2 per cent methyl linoleate failed to enhance resistance.

The dietary approach in the study of susceptibility has been exploited further in a series of preliminary reports. *Mycobacterium leprae* infection of rats and hamsters was enhanced on diets low in vitamin E and high in unsaturated fats (22). Conversely the resistance of chicks to *Salmonella gallinarum* was enhanced (23) on diets containing excess of all known required vitamins plus ascorbic acid. The reported retardation of tuberculous infection of mice by vitamin E does not appear to depend upon the lipotropic action of the vitamin (24).

The interrelation of geography, nutrition and disease has been reviewed for the South African area (25) in the same general terms which Dubos used in experimental tuberculosis and for which Lurie evaluated the relative importance of host constitution and virulence of the parasite. Acclimatization of mice to a simulated altitude of 20,000 feet for three weeks induced a lowered citric acid content of lung tissue which presumably reduced their capacity to synthesize influenza virus, thus tentatively offering a mechanism to explain a correlative increase in resistance to the virus (26).

Studies by Martin *et al.* (27 to 31) on succinic dehydrogenase activity in tuberculous animals have contributed to a further understanding of the events which Dubos (20) is studying by a different approach. Tuberculous guinea pig kidney homogenates possess a diminished succinate oxidation mechanism which is restored by the addition of extracts of normal kidney; the restorative factor has been identified with desaminocoenzyme A. Hemorrhagic shock and tuberculin shock, as well as tuberculous infection, all cause a depressed tissue succinate oxidation which may be the biochemical lesion induced by Dubos (20) in the animals maintained on altered diets. Such a lesion would have repercussions in many of the reactions contributing to defense and dependent upon a smoothly functioning coupling of oxidation and phosphorylation (28, 32, 33).

As a model of what may occur *in vivo*, the HeLa cell in tissue culture ap-

appears to be quite responsive to the exterior environment, assuming phagocytic properties towards *M. tuberculosis* when the tissue culture medium contains horse serum but not when human serum is employed (34). After the process of ingestion has occurred, the presence of either serum supports luxuriant intracellular growth of the bacilli, so that the horse serum apparently renders the HeLa cell more susceptible to the act of infection. This bears upon the different results which investigators obtain when studying monocytes from vaccinated and normal animals for their ability to retard intracellular growth of *M. tuberculosis*. A more exact index of the differences between the two kinds of cells may be provided by a measure of the ingestion phase. On the other hand, in this regard Raffel (35) very cogently points out the possibility that selection of the proper host cell population may be the key to a solution of this experimental problem.

Localization, dispersion and surface activity.—As a most important corollary to earlier studies on enhancement of infection by adrenalin and anti-complementary substances by Evans, Miles & Niven (36), the determinant aspects of the route of injection on lethal infections in mice was studied by Dutton (37). Eight of ten species of bacteria were less lethal by the intravenous route than by either the subcutaneous or intraperitoneal routes. This suggests that for the majority of the species, an early removal of bacteria from the subcutaneous tissues to the lymphatics and the blood stream may be advantageous to the host. Localization is likely to be an adequate mechanism for resistance when the dose is small and high virulence is not a property of the strain, or if the dose be large and a rapidly acquired humoral defense is induced. (For fuller discussion, see references 1 and 2.) Higginbotham & Dougherty (38) have also studied this question although their paper is more properly reviewed in a later section. The efficiency of filtration by the popliteal lymph node of the rabbit was studied on spores and vegetative cells of *Bacillus subtilis* and *Bacillus anthracis* (39). The two species were strikingly different in the ease with which they were filtered out; *B. anthracis*, especially in its vegetative cell form, was efficiently removed. This apparent adherence to cells of the lymph node calls to mind the earlier report of Falk (40) on the correlation between surface charge and virulence of pneumococci.

A further example of surface activity has been demonstrated by Duthie (41) in studies on the absorption of fibrinogen by coagulase-positive staphylococci, leading to aggregation of the cocci. The absorption is conditioned by a "clumping factor" in the cells, apparently distinct from coagulase. Free negative-charged groups on the absorbed fibrinogen are postulated to unite with positive-charged groups in the cocci leading to aggregation. The possible *in vivo* significance of this observation may well lie in the deviation of fibrinogen from its role as a pathway for leukocytic migration in addition to reflecting a purely aggressive function on the part of the cocci forcing localization and impeding dispersal.

The determinant action of lipotropic macromolecules on resistance to tuberculosis is reported to be a function of molecules with high lipophilic:

hydrophilic ratios (42, 43). Using a series of detergents prepared by forming polyoxyethylene ethers of a high molecular weight condensate of p-tert. octylphenol and formaldehyde, Lovelock & Rees produced definite suppression of growth of *M. tuberculosis* in the phagocytes of mice when the animals had been treated several days prior to exposing its monocytes to the facilli. The results with ingested Victoria Blue dye suggested that the cells had taken up a quantity of the detergent which had been uniformly distributed in the monocyte and made available for solution of the dye. In studies on thermal shock hemolysis Lovelock (44) showed that detergents with 10 to 20 ethylene oxide units greatly decreased the hemolysis by thermal shock, whereas those with 30 units slightly decreased it, and those with 60 units increased the damage. Now, turning to the effect on monocytes and their handling of tubercle bacilli, the 10 to 20 unit compounds were antituberculous, 30-unit compounds were inactive and those with 60 to 90 were enhancing as far as bacillary growth was concerned. Apparently the detergents concentrate *in vivo* within the monocyte, are able to solubilize lipids ingested by the cells, and can also adsorb or displace cholesterol preferentially from red cell membranes. Those active on the tubercle bacillus most likely displace the hydrophobic lipids preferentially rendering the bacilli more sensitive to intraphagocytic digestion. Enhanced enzymatic activity of the monocytes or of its parasite has been considered as an additionally possible mechanism.

Mixed infections.—A powerful tool in the form of germ-free animals has allowed experimentalists to study the determinant activities of multi-flora populations on such problems as amoebic dysentery and dental caries (45, 46, 47). Reyniers and his school have shown conclusively that the ability of *Entamoeba histolytica* to produce acute ulcerative amoebiasis in the guinea pig depends on the presence of other organisms in the gut, which share responsibility for the disease. In germ-free animals, not only were amoebic lesions absent but the amoebae survived poorly and were incapable both of independent survival in the intestine and of tissue invasion, suggesting that the secondary bacterial invasion is the determinant factor for the characteristic pathology of the disease. In the case of dental caries, Orland *et al.* (47) have demonstrated the bacterial origin of caries, and it remains now to learn which constituents of the diet predispose to caries, provided the organisms are present.

Hormonal determinants.—The large number of studies concerned with the influence of hormones on the various aspects of the infectious process continues unabated, and much of this is not pertinent to the review at hand. Four reports (48 to 51) of a more general review nature deal with stress, molecular mechanisms in inflammation and stress, the role of hormones in tuberculosis and problems involved in the bio-assay of anti-inflammatory substances.

Higginbotham & Dougherty (38) have studied a strain of staphylococci in which dispersal via the intravenous route of injection allowed the host animal (mice) to handle the infection completely whereas localization via

the intraperitoneal route of injection was rapidly fatal. Adrenalectomy reduced the resistance to the intravenous route by 1000-fold and accomplished this apparently by increasing the animal's sensitivity to the bacterial endotoxin, released during phagocytosis in the liver and spleen. Hydrocortisone antagonized the effect of adrenalectomy. A similar situation has been described by Payne *et al.* (52) wherein cortisone allowed growth and toxin production by normally avirulent strains of *Pasteurella pestis* to proceed to such an extent that the mice were killed by the toxin through the mechanism of so-called secondary shock. Specific immunization prevented this effect, by retarding the bacterial multiplication and subsequent toxin production.

In conjunction with tests on the ability of cortisone to enhance the incidence of bacteremia following intradermal inoculation of pneumococci in rabbits it has also been shown that the new drugs, chlorpromazine and methazine, act similarly but less extensively in their suppression of local inflammatory reactions. Such activities could not be replicated in mice although chlorpromazine did enhance infection in mice via the intranasal route (53).

The action of cortisone in enhancing susceptibility to plague toxin is paralleled by the ability of the hormone to affect young rabbits in such a way that they respond to a single dose of meningococcal toxin in a way normally requiring two injections 18 to 24 hours apart (54). This has been interpreted to mean that the hormone interferes with the protection against the vascular necrotizing action of the toxin afforded by the RES. Once a lethal dose of toxic material is accumulated by whatever the route of infection, the fate of the animal is determined despite the ultimate fate of the bacilli *in vivo*, which is a finding completely in agreement with that reported by Smith *et al.* (55 to 58) in a most significant set of papers on the pathogenesis of anthrax infection.

Frenkel (59) has studied the adrenal necrosis occurring in chronic toxoplasmosis resulting from selective parasitism of adrenal cells. This represents in some degree an enrichment culture aspect whereby endocrine factors immobilize the general immunity in a situation in which *Toxoplasma* and *Besnoitia* selectively parasitize tissues surrounding areas of steroidogenesis.

The somatotrophic hormone appears to antagonize infectious processes, facilitating connective tissue proliferation by an effect on permeability relations in the tissues. The hormone significantly increases the LD₅₀ value of *Salmonella typhi* in mice, but does not affect the LD₅₀ value of the endotoxin (60). This may indeed be related to the striking rise in complement titer induced by the hormone itself or by contaminating thyrotrophic activity (61).

Work on the role of sex hormones and their mediating effects in infection is increasingly accurate and quantitative.

Lamming *et al.* (62, 63, 64) rapidly established *Corynebacterium pyogenes* in the bovine uterus during the luteal phase but failed to do so during the oestrus phase. In ovariectomized cows the lowered resistance was shown to be the result of the action of progesterone, whereas endogenous or exogenous

oestrogen maintained the uterine mucosa in a state highly resistant to infection, presumably by increasing the terminal blood supply to the mucosa with the attendant readily available defense mechanisms. Some light has been cast upon this phenomenon of enhanced susceptibility of the uterine mucosa by studies of West & Cervoni (65) in terms of the uterine glycogen content under ovarian hormone administration. The nature of the changes in terms of altered glycogen content of defense cells recalls the hypothesis and experiments of Dubos (20) on susceptibility to tuberculosis discussed earlier. This entire subject of altered susceptibility as a result of hormonal changes within the framework of the altered physiology of pregnancy has been reviewed in relation to poliomyelitis attack rates and virus diseases in general (66, 67). The subject of adrenal cortical physiology in disease is reviewed by Knowlton (68).

As an *in vitro* model of host-parasite relationships under hormonal control Kilbourne has uncovered what may essentially be a multiplicity reactivation phenomenon to account for the reactivation of noninfective influenza in cortisone-injected eggs (69). It is postulated that cortisone negates the auto-interference phenomenon of noninfective virus upon reproducing virus and mediates the hexose-6-phosphate level to allow degraded virus to reactivate. Manipulation of the time at which cortisone was injected into the embryonated egg led to prolonged survival of the embryo and attainment of higher virus yields, depending on the time of administering the hormone (70). Other investigations in which the parameter of time was also used to mediate the result have shown that cortisone, added to HeLa cell cultures, can affect the cytopathogenicity induced by Types I and II poliomyelitis virus in opposite ways, depending on the time at which the drug is administered in relation to the time of infection (71).

Mucins and bacterial polysaccharides.—Our understanding of the phenomenon of enhancement of infection by polysaccharides, mucopolysaccharides and related compounds has been extended by the work of Shilo *et al.* (72) and Davies *et al.* (73) who have shown that the intravenous inoculation of bacterial levans into rabbits prevented the later diffusion of trypan blue into sites of skin injury and also prevented in guinea pigs the neutralization of diphtheria toxoid injected intradermally by circulating antitoxin. The observations are explained in terms of the entry of large levan molecules into the interstices of capillary endothelium, thereby limiting permeability and inhibiting the inflammation process. These experimental observations and conclusions confirm and extend earlier studies (74 to 77) on the mechanisms by which hog gastric mucin has been postulated to act. Additionally, mechanisms derived from the papers of Smith *et al.* (78 to 81) on the identification of the enhancing factors of mucins, confirmed in substance by Walton & Taylor (82) relate to: (a) the anticomplementary nature of the heparin fraction of the mucin (as well as of dextran sulfate); (b) the viscosity-contributing effect of the C polysaccharide fraction, this substance acting synergistically with the heparin fraction and possibly mediating capillary perme-

ability; and (c) the particulate residue fraction which acts in concert with the C polysaccharide to enhance infection independently of the heparin fraction. The mechanism of action of all these substances needs reconsideration in their details in view of the development and study of serum properdin (*vide infra*). As Smith has pointed out so cogently a decision as to the mechanism of actions of such complex substances as mucin, and it should be added, of the dextran and levan preparations, if only because of their molecular weight inhomogeneity, is impossible in view of the diversity of biological activities each preparation manifests.

The role of altered capillary permeability remains an ever continuing problem for resolution in these phenomena in the light of the enzyme-like globulins uncovered by Miles and his colleagues (83, 84) in serum. These materials, discussed in a later section of this review, appear to be of such physiologic importance in their histamine-like activity that their activation by reactions other than dilution and aging of serum clearly calls for examination. The aspect of their nature and reactivity which bears upon the infection enhancement phenomenon (induced by mucins) lies in the speed with which they can be activated. Thus, it is of interest to learn whether the mucins and related substances act as liberators of such substances and thereby affect the early stage of bacterial invasion, perhaps complementing the type of action which adrenaline effects during the first hours of infection. The immediate response which infection induces in the plasma-leukocyte migration system (85) (irrespective of which of the two components or both are directly affected) also suggests that a mechanism by which capillary permeability can be altered significantly, albeit temporarily, would permit an increased concentration of plasma migration factor (86) or intracellular activator of leukocytic migration (85). Thus a variety of seemingly independent phenomena could be brought into relationship via an adjustment of vascular permeability. An excellent discussion of the role of mucin and the protection of the body has been given in the Croonian lecture by Florey (87).

Studying the inorganic elements for their role in the biology of infections, Phillips (88) has demonstrated that a mixture of cobaltous ion and the tetrasodium salt of the chelating agent, ethylenediamine tetraacetic acid, act additively to reduce the survival time of mice infected with a Group A streptococcus, equivalent to a 20-fold reduction in the LD₅₀ value. The mixture caused a significant reduction in the phagocytic ability especially of the mononuclear portion of the peritoneal exudate and permitted almost unrestricted growth of the streptococci *in vivo*. The interesting aspect of this work was the apparent specificity of the cobaltous ion.

The irradiation pattern as a determinant of resistance.—This subject has been extremely lively, and thoroughly reviewed (89, 90). However, there are certain aspects particularly pertinent to the subject of this review not covered elsewhere. The histopathology of bacterial infection in irradiated mice has been shown by Congdon and co-workers (91) to lack the element of purulent exudation in the inflammatory lesions. Bacteria are seen in the injured in-

testinal epithelial cells and necrotizing bacterial ulcers. Growth of microorganisms may be seen along the vascular channels or through the vessel walls into adjacent tissues. Parallel results using the culture method were obtained by Gordon *et al.* (92). This is the basic lesion of the irradiation infection and reparative processes are absent. If the cellular elements of inflammation are especially stimulated by injections of particulate matter, 44 to 74 microns in diameter, then Smith *et al.* (93, 94) were able to observe increased survival time of irradiated mice, which were succumbing to *Proteus* and *Escherichia coli* infections. Resistance to pseudomonas infections was not so enhanced, apparently attributable to the rapidity with which this infection generalizes in irradiated animals in advance of the cellular stimulation afforded by the particulate matter. On the other hand, Smith *et al.* (95) failed to obtain evidence that increasing spleen weight led to increased ability of that organ to remove bacteria from the circulation in irradiated mice (cf. 96). Hammond *et al.* (97) observed that irradiated mice permitted organisms introduced via the oral route to establish themselves to a greater extent than nonirradiated animals and confirmed Dubos' observation (20) on the effect of a 24 hr. fasting period prior to infection. Animals so fasted, but not irradiated, imitated the results in the irradiated, unfasted group.

Marcus *et al.* (98) found that irradiation decreased the ability of antibiotics to combat lethal infections. One interpretation of this type of result is that animals by virtue of irradiation may be rendered more susceptible to bacillary toxins liberated as a result of action of the antibiotic on the organisms *in vivo*.

ADAPTIVE PATTERNS OF RESISTANCE

Cellular patterns.—The cellular defenses are among the most plastic and adaptive to invasion by bacteria. The studies of most significance in the period under review are those which have quantitatively evaluated the response of the RES to injection of indicator substances before, during, or after the exposure of the host to *Salmonella* endotoxins and histamine-liberators. In the first category, Biozzi, Benacerraf & Halpern (96) have reported that 10 hours after an intravenous inoculation of dead *S. typhi* or Boivin antigen into rabbits the phagocytic index of the liver and spleen is maximally depressed, whereas a second dose given two days later stimulates these organs in terms of phagocytic activity per unit weight of liver and actual increase in weight of the organs. A single subcutaneous injection, however, of the same materials stimulated the RES at once, contrary to the depression induced after the first intravenous injection, but the same hyperactivity of the RES ultimately resulted by either route. Cortisone (1 mg. per day for six days) largely inhibited the stimulatory effect of multiple injections of the antigens. Although there is no direct evidence presented, this suggests that the RES does participate in the defense against the endotoxins of the gram-negative group, as reviewed thoroughly by Thomas (99). Benacerraf *et al.* (100) have shown that an increase in blood flow through the

liver was followed first by increased phagocyte activity and second by hypertrophy of the organ. The presence of an endogenous plasma factor in rabbit plasma one hour after injection of bacterial lipopolysaccharide appears to exert a stimulatory effect on human leukocytes in their phagocytic activity (101, 102). The plasma factor appears coincidentally with the onset of leucopenia and may well be derived from the blood cells, for dog leukocytes have also been shown to produce a phagocytosis-augmenting factor (103). The ribonucleic acid and deoxyribonucleic acid nature of this substance has been postulated (104).

Extending the original observation of Jancsó on the role of histamine as a physiological activator of the RES, Gözsy & Kató (105) have shown that derivatives of the bicyclo [0.3.5] decapentaene skeleton as histamine liberators induced intense phagocytic activity by endothelial cells of vessels in the skin, an effect antagonized by antihistaminic substances. The role of azulene compounds in the prolongation of survival time as well as antituberculosis effects measured in terms of Feldman's indices of infection in guinea pigs infected with the bovine strain has also been explained by Kató & Gözsy in terms of their histamine-liberating effects and consequent stimulation of the RES (106, 107, 108).

Reports on the effect of cortisone, ACTH and adrenalectomy on phagocytosis continue to be somewhat conflicting, depending mainly for this on differences in dosage and time of treatment. Ludányi *et al.* (109) observe with cortisone doses of 20, 12.5 and 12.5 mg. on three successive days a depression of phagocytic activity of rabbit blood cells towards staphylococci and *Salmonella*. Similar trends were produced by 15 units ACTH 24 hr. after injection. Crabbé, however, emphasizes (110) that it is the cell component which is affected inherently by cortisone and ACTH rather than the serum in phagocytosis. But it is difficult to accept this conclusion in view of the mild methods used to separate cells from serum in much of this work, a notable exception having been earlier cited (103). The breakdown of the cellular element in cortisone treatment and irradiation appears to have been pinpointed to the phase of intracellular digestion (111).

The participation of the polymorphonuclear neutrophile in the generalized Schwartzman reaction has been discussed in a general review (99). The ability of three synthetic polymers (sodium polyanethol sulfonate, dextran sulfate and sodium polyvinyl alcohol sulfonate) to act as adjuvants with bacterial endotoxins in the production of the generalized Schwartzman reaction indicates that the polymers substitute for a mechanism normally provided by the leukocyte (112). The mechanism as postulated (113) is that the stage of preparation for the Schwartzman reaction (the first 24 hr. after first injection of toxin) may involve a reaction with leukocytes leading to the availability of a material with the biological function of the polymer, which is to precipitate fibrinogen (as fibrinoid) when polymer-precipitable fibrinogen appears in the blood in response to the second injection of the toxin. According to Stetson (114) this reaction may in its general outlines be consid-

ered operationally analogous to the allergic response of sensitized rabbits. Such an hypothesis at least in its early statement brings some degree of unity to the diverse reactions one arbitrarily may include in the constellation of nonspecific resistance. Thus Stetson suggests that the generalized Schwartzman reaction can be conceived as an example of "natural hypersensitivity."

Mucoproteins and mucopolysaccharides.—In the light of increasing knowledge on the anticomplementary action of heparins and mucopolysaccharides and their increasingly prominent role as mediators of capillary permeability, it is pertinent to mention papers on the metabolism of the family of heparins and mucopolysaccharides. Dorfman has reviewed the subject in general (115), and Loeven (116) has discussed the bondings between collagen and mucopolysaccharide in connective tissue. Although little is known of the significance for resistance of the ebb and flow of respiratory, gastric and urinary mucoproteins and mucopolysaccharides, the biophysical data which Tamm *et al.* (117, 118) have presented for urinary mucoproteins give valuable information on this class of compounds, interesting potentially from the standpoint of resistance. The appearance of these substances in the urine is affected by the parathyroid (119). The further demonstration that virulence-enhancing polysaccharides are not active as inhibitors of viral hemagglutination appears to separate sharply these two biological functions of such substances.

Increasing attention is being given to the behavior of the serum mucoprotein concentrations under various stimuli, but at present little can be said other than that, for example, in *Brucella suis* infection in guinea pigs, increases in this component are correlated in time with antibody formation (120). The homogeneous mucoprotein of serum has also been claimed to be responsible for the elevation of total serum polysaccharide and glucosamine in the inflammatory state (121). The RES has been implicated in the metabolism of mucoproteins inasmuch as it is lacking in cells of transudates but present in exudate cells, the blood monocytes giving a staining reaction indicative of this class of compounds (122). "Stress" lymphocytes appear to be rich in their content of mucoprotein, whereas eosinophiles and polymorphonuclear neutrophils give no such staining reaction.

The properdin system.—The most potentially far-reaching discovery in the experimentation on nonspecific patterns of resistance during the period under review is that of Pillemer and his colleagues (123) on the properdin system. The term, derived from the Latin *perdere*, meaning to destroy, refers to a substance isolated as a new serum protein. The properdin system is composed of (a) properdin which can be isolated from normal serum by absorption onto zymosan at 17°C. followed by elution, (b) complement and (c) Mg^{++} . Properdin is present in normal human and other mammalian sera. The properdin system (a) kills bacteria such as strains of *Shigella*, *Pseudomonas*, *Proteus*, *Salmonella*, *Para-Colobactrum*, and *Escherichia* (124); (b) neutralizes Newcastle disease virus; (c) lyses erythrocytes from cases of paroxysmal nocturnal hemoglobinuria and tannic acid-treated red blood cells

in the absence of antibody; (d) may be of importance in the collapse of resistance to infection and endotoxins in hemorrhagic shock (125, 126, 127); and (e) protects rats and mice significantly against LD₁₀₀/30-day and LD₁₀₀/6-day doses of irradiation (128, 129).

Properdin reacts with bacterial cell walls from yeasts and gram-positive and gram-negative bacteria, as well as with bacterial endotoxins (130). It also combines with high molecular weight native dextrans, native levans (except levan II), yeast glucan, the specific-soluble substance of Types XIV and IV pneumococci, and hog gastric mucin. This suggests an additional role for hog gastric mucin in the enhancement of infections (72 to 81) and further helps to elucidate the mechanism whereby cell wall preparations of *E. coli* induced mice to die of infections by avirulent *E. coli* strains (131), since these preparations inactivate properdin. The increased susceptibility of sublethally irradiated mice to *E. coli* and avirulent *P. pestis* infections (132, 133) is open to interpretation as an example of reduced tolerance to endotoxins resulting from depressed properdin titers.

Initially, properdin was shown to undergo a rapid fall in titer within 1 to 2 hr. after the inoculation of zymosan and then to rise markedly in 2 to 14 days (134) to 200 to 300 per cent of normal. This helps to explain Rowley's observation (131) that mice given zymosan were immediately susceptible to avirulent *E. coli* infections whereas later, in the "properdin-rebound" phase, the mice resisted virulent strains of *E. coli*.

Pillemer *et al.* (123) initially demonstrated properdin (P) as the serum factor necessary for inactivation of C'3 by zymosan (Z) at 37°C. At 15°C. no inactivation occurs although zymosan forms an insoluble complex, PZ, with properdin, leaving the serum deficient in properdin (RP). Addition of Z to RP does not inactivate C'3, but addition of P leads to full inactivation. P is therefore essential for the reaction: C'3+Z. The reaction P+Z requires C' and Mg⁺⁺, ionic strength less than 0.4 and a temperature greater than 10°C. Properdin appears to be a euglobulin with an isoelectric point of 4.8 to 6.5, a sedimentation constant of 27S, and is resistant to 66°C. for 30 min. although it is destroyed at 100°C. in 5 min.

Properdin is measured by the addition of varying amounts of test sample to a constant amount of RP, incubated at 37°C. for 1 hr. with a standard amount of zymosan. The fall in C'3 is measured; 1 unit of P is equal to that amount which reduces C'3 of RP from 120 units to zero (123, 135).

An interesting problem in comparative immunology is thereby posed by Briggs who observed that the late fourth and early fifth instar larvae of *Bombyx* are greatly enhanced in their susceptibility to avirulent strains of *E. coli* by the inoculation of the larvae with zymosan (136). It would be most significant to establish the existence of a properdin or properdin precursor system in the hemolymph at this level of the animal kingdom.

Tissue and serum factors.—*In vitro* studies on the growth of Type I pneumococci in whole blood and serum of guinea pigs suggests that the bactericidal action can be affected by the pH of the mixture during growth which

in turn may be related to a change in the sulfhydryl content as a result of alteration of the CO_2 balance (137). The net effect has been the *in vitro* production of a new electrophoretic component from the albumin fraction (138). The *in vivo* significance of this pattern of events remains to be determined. Pneumococcal extracts have been obtained from the liver and spleen of rats and guinea pigs, the increased activity of the rat preparation on purification suggesting to the author the existence of a possible natural antagonist in the tissues, not observed in the guinea pig (139). Murayama (140) has observed activation of adenosinetriphosphate (ase) of leukocytes by Type I pneumococcus in the resistant guinea pig as contrasted to the inhibition of the enzyme in similarly prepared nuclei-free homogenates of leukocytes of the susceptible rat. The significance of these results for an understanding of events in actual infection would seem to depend on a demonstration that the enzyme in the intact cell during successful infection is available to pneumococcus action in the susceptible species.

No additional information has been presented to evaluate the significance of plasma factors reported to be involved in the acceleration and inhibition of migration of human leukocytes *in vitro* (86). The accelerating factor resides in Fraction II, whereas the inhibiting factor(s) reside in III and IV+V. The discrepancy between the results in this study and those reported by others (85, 141, 142) is essentially a question of the role of the plasma in the phenomenon of leukocyte migration in humans contrasted to the laboratory rodent.

The resolving aspect to the difference in results obtained may well lie in the observations that plasmas vary so widely from person to person in their accelerating property (86). Variability in the response of animals was observed (85) but was concluded to be an inherent property of the migrating leukocytes peculiarly susceptible to "stressing" reagents.

The participation of serum antibacterial factors in resistance reactions is certainly not yet clear. The serum bactericidal substance for *B. subtilis* differs from lysozyme in certain respects (143) and is present at its greatest amount in the acute phase of various illnesses.

The proteinases of the bovine lung described by Dannenberg & Smith (144) catalyze the synthesis of polypeptides from certain amino acid esters and amides and appear to be derived from the mononuclear phagocytes. This suggests that such cells may be the source of the antibacterial peptides characteristic of the inflammatory exudate. Further, it raises the question of just how much of a response provides survival value to the host insofar as producing detestable amounts of these substances. Certainly when time is an important parameter of the constellation of patterns we call resistance, the enzyme-like globulins (83, 84) from serum which reproduce the vascular phenomena of inflammation are of interest but as Miles, Wilhelm & Mackay point out (83, 84), it is not known whether they are actually required in the dual role of determinants of, and adaptations to, resistance and infection. Miles *et al.* (83, 84, 145) have uncovered globulins in the serum or plasma of

guinea pigs normally in an inactive form, which can be activated by dilution or by aging of the serum. These globulins cause an increase in capillary permeability within 3 min. of intracutaneous injection. The permeability returns to normal in 15 min., thereby leaving a refractory state for 1 hr., which returns to normal in 5 hr. The globulins, although histamine-like in action, are not reversed in this action by mepyramine. The globulins are susceptible to inactivation by soy bean trypsin inhibitor; thus they resemble proteases. However, they are inactivated by sodium salicylate which also inactivates fibrinolysin. Their other activities include increasing the stickiness of the vascular endothelium and promotion of exudation.

The permeability factor which is activated on dilution is associated with the α_2 globulin fraction (its normal inhibitor is an α_1 globulin), and on a weight basis is about 1000 times more active than histamine. On this basis, it may be assigned tentatively the role of the natural mediator of capillary permeability changes. Its "substrate" and precise site of action is unknown (84). Associated with these globulins is a natural inhibitor substance. The source of the latter may be the eosinophilic leukocyte (152). Histamine and histamine-like physiological patterns recur in other aspects of resistance. For example, an adaptive pattern of response is induced in animals by injections of *Hemophilus pertussis* vaccine (146, 147, 148). The responses have included increased sensitivity to histamine and *Shigella* endotoxin, increased susceptibility to influenza virus (149) and increased sensitivity to irradiation (150). In the latter case neither neoantergan nor hydrocortisone prior and subsequent to irradiation affected the sensitivity to histamine, but this is subject to some degree of reservation since the author did not provide evidence that the animals were actually antagonized to histamine sensitivity during antihistaminic treatment.

Obviously it is at this time difficult to appraise the significance of increased histamine sensitivity in various conditions for its role in resistance in the absence of precise information on the target of histamine activity. In this regard it has been postulated that histamine reacts with phosphoric acid esters to form a ring structure on the ester to diminish thereby the surface tension properties of the ester (151). Knowledge of the strategic location of such esters in the vascular system would be invaluable.

Both polyvinylpyrrolidone and dextran have been charged with the ability to release histamine, acting on various animal hosts in a manner specific for the host (153). Thus, dextran affects the rat, whereas polyvinylpyrrolidone is most effective on the dog, producing in each case symptoms resembling anaphylactic shock referable to fundamental changes in capillary permeability. As others have also observed, the effect of histamine-liberating substances is often more severe than that of histamine injection due probably to the increased concentration of histamine at the cellular level.

The concept that acidic polymers of pathogenic bacteria may also function to neutralize lysozyme and other antibacterial substances in normal

tissues has been proposed by Skarnes & Watson (154) as a result of their observation that the glutamyl polypeptide of *B. anthracis*, hyaluronic acid, pneumococcus polysaccharide, Vi antigen and deoxyribonucleic and ribonucleic acids all inhibited lysozyme activity.

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THE BIOLOGY OF THE CELLULAR SLIME MOLDS¹

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INTRODUCTION

At present there are, to my knowledge, five laboratories throughout the world engaged in studying the cellular slime molds. In this circumstance, an annual review of the combined efforts of these investigators would be a very comfortable task but one that could carry little information to the reader. Instead, because of the above, and because the slime molds are a group little known to most microbiologists, I have chosen to increase the number of years under review and to discuss the general biology of this group in as much detail as spacial constraint permits.

In this, the work of recent years has been given major attention. Other reviews by Raper (41, 44), Bonner (2, 10), and Sussman (66, 67) have covered earlier investigations in detail or else have been focused exclusively upon the intriguing morphogenetic properties of the group.

The cellular slime molds stand at the boundary between the true Protista and the Metazoa and Metaphyta. During vegetative growth, the unit of existence is a single, amoeboid, phagotrophic cell, the myxamoeba. After the logarithmic phase has passed, the heretofore separate cells cooperate to construct an organized multicellular fruiting structure with morphologically differentiated tissues that appear metaphytic in character. Spores are then cast off, and upon germination, reassume the protistal condition. Figure 1 is a summary of the life cycle. Figures 2 and 3 illustrate the morphogenetic sequence that leads to fruit construction.

GROWTH AND NUTRITION

Myxamoebae are phagotrophic organisms that normally feed upon bacteria. The latter can serve as the sole source of nutrients (R. Sussman, unpublished data) and thus the problem of growing the slime molds in two membered cultures is reduced to one of providing good conditions for the growth of the bacterial associate while protecting the myxamoebae from the products of bacterial metabolism. The morphogenetic sequence introduces a complication, since the conditions that permit optimal growth of the myxamoebae do not necessarily allow optimal fruiting and can, in fact, seriously interfere with that process (19, 36, 37). A start has been made in the direction of axenic culture. A defined medium has been devised but as yet not a synthetic one.

¹ Previously unpublished experimental data included in this review were obtained from investigations supported by funds from O.N.R. and N.I.H. The survey of literature was completed in February, 1956.

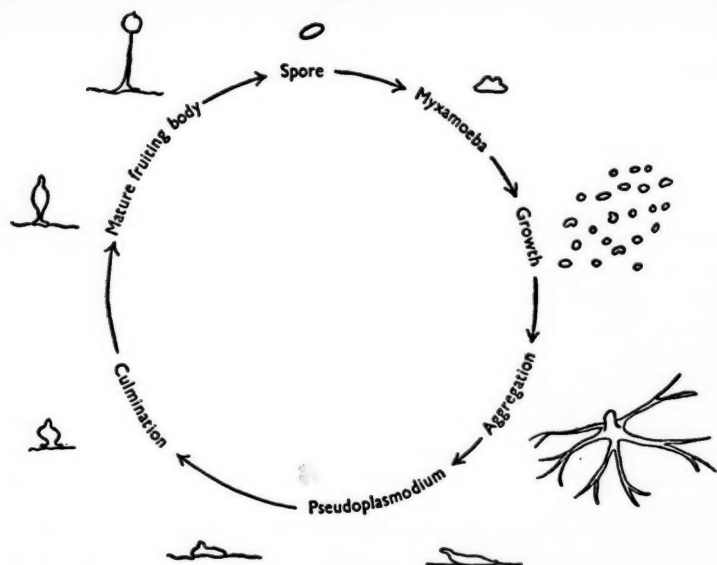


FIG. 1. A schematic diagram of the life cycle of *Dictyostelium discoideum* (61).

MASS AND CLONAL CULTURE

For stock plates, two to three spore masses or a loopful of myxamoebae can be spread on agar with a few drops of bacterial broth culture. The associates generally employed are *Escherichia coli* and *Aerobacter aerogenes* and the medium is that of Bonner (4), containing glucose, peptone, yeast extract and mineral salts (hereafter called SM) used straight or diluted. Upon incubation at 22°C., ingestion of the bacteria is complete by about 40 hours, and the fruiting process occupies an additional 24. For special purposes (49) a plain agar substratum can be employed. Where the fruiting process is to be observed directly on the growth plate, it is sometimes advisable to make an "X" streak of bacteria on dilute medium and inoculate a small number of spores or myxamoebae at the center (44).

Clonal growth can be achieved by delivering and spreading aliquots containing a few spores or myxamoebae with bacteria (55, 56). Plaques appear after 3½ days within the bacterial growth area. Normal fruits are formed in the ensuing 24 hours. The efficiency of plating (plaque count/direct count $\times 100$) is about 70 per cent, although with log phase myxamoebae it can reach 90 to 100 per cent (58, 68). The plaque morphology is distinctive and heritable. Up to 150 plaques per plate can be scored conveniently for count or appearance.

A study has been made of the growth kinetics of *Dictyostelium discoideum* from a mass inoculum of 10^8 spores per plate with *A. aerogenes* on SM medium and with a concentrated suspension of washed bacteria on non-nutrient agar (68). Spore germination and the lag phase required about seven hours on the former and ten on the latter. The log phase generation times were approximately the same (ca. 3.3 hours). At the stationary phase the counts were 10^9 myxamoebae per plate (9.5 generations after 44 hours) and 10^7 (3.8 generations after 24 hours) respectively.

INGESTION OF BACTERIA

This topic has been the subject of a number of conflicting reports by earlier workers. Potts (33) concluded that bacteria were digested extracellularly by *Dictyostelium mucoroides*. It has been suggested (52) that the slime mold-bacterial relationship is mutualistic. However, I think that anyone else viewing the damage that myxamoebae can wreak upon a bacterial colony would agree that the relationship is just about as mutualistic as that more celebrated one between the little Oysters and the Walrus and the Carpenter (18). Raper (36) observed *D. discoideum* feeding upon *Bacillus megaterium*, and his camera lucida drawings indicate that the myxamoebae ingested the bacteria by invagination, one of the usual methods employed by the Sarcodina (28). After ingestion, vacuoles were formed and the bacteria completely digested. Bacterial spores were also taken in but eliminated without decomposition.

RANGE OF UTILIZABLE BACTERIA

The myxamoebae will eat practically any bacterium except rough or exceedingly mucoid varieties (36). *Dictyostelium discoideum* can grow adequately upon a large variety of saprophytes including representatives of Enterobacteriaceae, Pseudomonadaceae, Micrococcaceae, Rhizobiaceae, et al. (36). Pathogenic species also serve as food sources (38). Singh (51) reported that *D. discoideum* and *D. giganteum* would grow well in sterile soil to which a variety of soil bacteria had been added.

Certain bacteria have been found to be useful associates for special purposes. Thus *Serratia marcescens* is used to vitally stain *Dictyostelium discoideum*, *D. purpureum* and *Polyspondylium violaceum* since these species fail to digest the red pigment (42). *B. megaterium* because of its size, is useful in studying the process of ingestion (36).

COMPLEXITY OF THE MYXAMOEBA-BACTERIUM RELATIONSHIP

The ecological relation between myxamoebae and bacteria is not exclusively one of predator and prey but also involves competitive interaction for dominance in the niche. This can be demonstrated convincingly in broth cultures. Consider the following data (R. R. & M. Sussman, unpublished data). (a) Inoculation of slime mold spores or myxamoebae and *A. aerogenes* into SM broth is followed in a matter of hours by degeneration and disappearance of the slime mold. If the number of bacteria introduced is progressively

decreased progressively greater periods of transitory myxamoeboid growth occur, and degeneration is increasingly delayed. (b) Inoculation of spores or myxamoebae into a suspension of washed *A. aerogenes* in buffer permits adequate growth and serial passage of the slime mold. (c) Inoculation of spores or myxamoebae into SM medium with a chromogenic pseudomonad isolated from soil (whose growth rate in glucose peptone broth is quite low) allows luxuriant growth of the slime mold.

These results point clearly to the existence of competitive interactions between predator and prey; the outcome is decided by their relative generation times.

Competition between myxamoebae and bacteria also occurs on agar, and different species display a variety of reactions. Certain isolates from nature can grow on *E. coli* but not *A. aerogenes* and vice versa (R. R. & M. Sussman, unpublished data) and some can grow well in mass platings on SM with *A. aerogenes* or *E. coli* but form very small plaques or none at all when plated clonally. In the latter cases, dilution of the medium permits good plaque formation.

The influence of constituents of solid media upon growth and fruiting has been extensively studied (37). Of the media employed, a dilute carbohydrate-peptone agar, hay infusion agar and carrot infusion agar yielded good growth and morphogenesis. Other factors such as pH (optimal range = 6 to 6.2) and temperature (optimal range = 20 to 25°C.) were examined. Humidity also affects both motility of the cells and the morphogenetic processes (37, 51).

A general principle of cultivation can be inferred from the foregoing data. The optimal conditions for growth and morphogenesis are those which provide a balance between the growth kinetics of the myxamoebae and bacteria. If the advantage is on the side of the myxamoebae they will be able to grow and fruit before the bacteria can pour out metabolic waste products sufficient in kind or quantity to stop either process. Increasing the advantage can only diminish the net amount of consumable bacteria and thus cut down the yield of myxamoebae. Decreasing the advantage interferes successively with the ability to fruit and to grow.

AXENIC CULTURE

Myxamoebae can be grown on killed *E. coli* or *A. aerogenes* and in fact can be carried routinely in this manner if need be. Raper (44) grew *D. discoideum* through seven serial passages with autoclaved, 50-fold concentrated *E. coli* on lactose-peptone agar. Although the growth rate was considerably lower than that obtained with living bacteria, normal morphogenesis was obtained. No data were given concerning comparative cell yields. R. R. Sussman (unpublished data) modified this procedure in the light of investigations (14, 62) which have provided a defined (but rather tediously prepared) medium. The bacteria were washed by centrifugation, disintegrated either by grinding with powdered dry ice or by treatment in a blender with ballotini beads, leached of free amino acids by water extraction at 70°C. and of nucleotides

by repeated 1 *N* sodium chloride extractions at room temperature, concentrated 50-fold, and autoclaved. The resulting suspension was spread on washed agar containing riboflavin, thiamin and a mixture of amino acids. Growth of *D. discoideum* and *D. mucoroides* proceeded at a rate and with a yield comparable to those obtained with equivalent amounts of living bacteria, and fruiting was normal. Clonal growth could also be achieved; coupled with the fact that a sufficiently minimal nutrient assembly had been supplied, this made it possible to isolate a few auxotrophic mutants after ultraviolet irradiation.

The defined medium (14, 62) contains beef extract (replaceable by a mixture of purines and pyrimidines); a combination of vitamins, of which riboflavin and thiamin were found to be absolutely required; yeast extract; salts; and glucose (used not as a carbon or energy source but for the synthesis of polysaccharides necessary to fruit construction). Upon this medium was spread an autoclaved paste containing a purified protein fraction from *A. aerogenes*. The fraction was obtained from ground cells by extraction at pH 10 and precipitation at pH 7. This was followed by treatment with organic solvents and then repeated alkaline extractions and neutral precipitations. Material taken from successive solubility partitions gave constant growth activity per milligram of dry weight, had a constant elementary composition (C, H, O, N) and showed in the ultracentrifuge a single component whose molecular weight lay between 25,000 and 40,000. The elementary formula suggested polypeptide composition and bioassay of HCl hydrolysates showed the presence of 17 amino acids. The material was found to be unstable and soluble at pH 4 and 9.5, stable and insoluble at pH 7. This fraction has been obtained from three gram-negative bacterial species in which it comprises 5 to 6 per cent of the dry weight of the cell. It has not been found in gram positive bacteria or in any of a number of plant and animal tissues. Autoclaved pastes of gram positive bacteria permit extensive growth, but the active fraction is not extractable by any means used thus far. Autoclaved pastes of other forms, including yeasts and higher plants and animals, were totally inactive.

The purified protein fraction was found to have at least two functions. One was to serve as a carbon and energy source, but this requirement could also be satisfied by proteose-peptone or gelatin. The second function is unidentified at present and no suitable replacements of the bacterial protein have yet been found.

The need for a growth factor that is stable only when insoluble is consistent with the phagotrophic pattern of the myxamoebae. In the pH range over which myxamoebae can grow, the protein factor could be found only within living bacteria or the solid debris of dead ones.

DEVELOPMENTAL PHYSIOLOGY

The most interesting aspect of the slime molds is their developmental sequence. Three stages are generally specified: aggregation (Fig. 2), in which

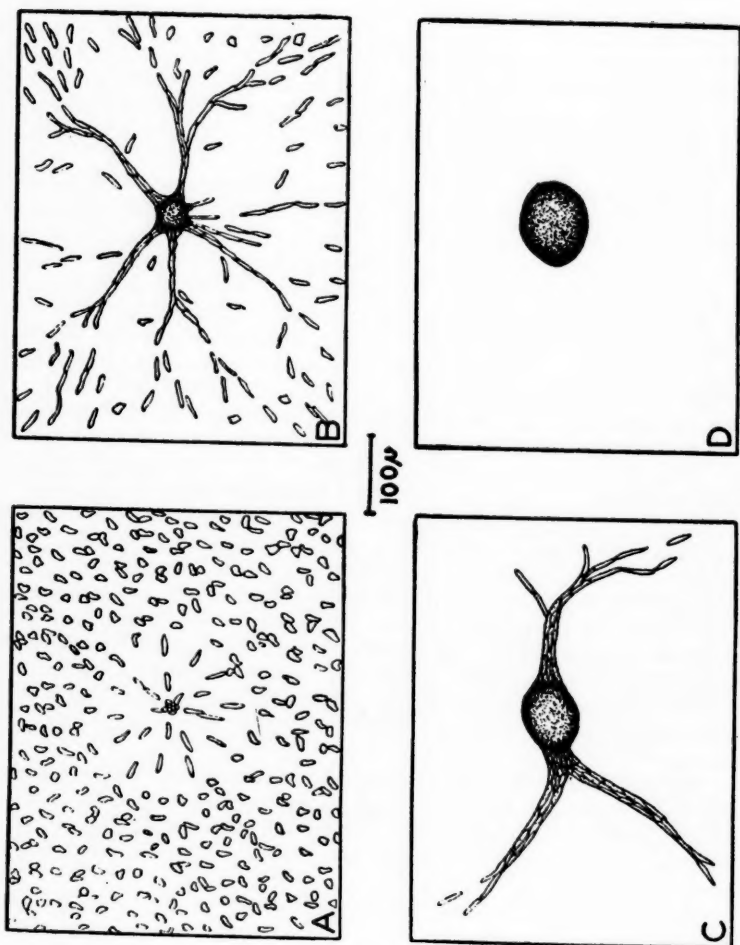


FIG. 2. A semi-diagrammatic illustration of the aggregation of myxamoebae (2).

the separate cells enter into a multicellular mass; pseudoplasmodium formation (Fig. 3), in which the conical aggregate is transformed into a cigar-shaped structure, endowed in the case of *D. discoideum* with the capacity to migrate over great distances; and culmination (Fig. 3), in which an organized fruit or sorocarp is erected by morphogenetic movements. A summary of the main physiological information pertaining to these stages follows.

AGGREGATION

After the myxamoebae have entered the stationary growth phase, the first sign of impending aggregation is a sudden drastic elongation of the cells and increase in pseudopodial activity. This is followed by rapid alignment in a radial pattern, generally a few hundred micra in diameter. The cells then begin to move toward the aggregative center coalescing into ramified streams of separate individuals as they do so. Cells distal to this pattern are subsequently alerted and move radially toward the center or tangentially to an established stream. The streams enlarge and branch in this manner so that the wheel-like pattern at its peak can encompass a diameter of about 0.5 mm. The arrival of cells at the center of aggregation results in the formation of a conical mound which quickly assumes the typical pseudoplasmodial shape. The foregoing description holds only for relatively low cell densities. On growth plates, where the density can be enormous, one sees only a blocking out of mounds by concerted radial motion of the cells within small areas and without the formation of extensive streams and branches.

Several factors that play major roles in causing and regulating aggregation have been identified. The attraction of outlying cells to the aggregative centers is known to be chemotactic. The attracting substances are produced at the center and along the cell streams (4). The aggregations are species specific (42), at least in part due to differences in the chemotactic agents (48). The number of aggregative centers formed depends critically upon the population density and the total number of cells (57). Each aggregate requires for its evocation, the presence of an initiator cell that extends the initiative stimulus to its neighbors, the responder cells (57, 58).

Involvement of chemotaxis.—Although a chemotactic mechanism of aggregation was long suspected (1, 33, 39), it was not until 1942 that supporting evidence was obtained. Runyon (47) showed that if myxamoebae were allowed to commence aggregating on one side of a cellophane membrane, cells placed on the other side would become oriented and aggregate in coincidence with the center and streams of the former. Subsequently, a very elegant demonstration was devised by Bonner (4). Myxamoebae adhering to a cover-slip under salt solution were placed in a position facing a well-established center on another cover-slip. Within five minutes, they were induced to begin aggregation, and the induced center was invariably situated at the minimal possible distance from the pre-established one. The influence was detectable up to a distance of 800 micra through the salt solution. The same type of reaction has been observed across thin agar membranes (65), but is limited to

a distance of two hundred micra or less. Bonner also showed (4) that if a slow current of salt solution was allowed to pass over myxamoebae that were beginning to aggregate, cells down-stream from the aggregative center could respond and would move towards the center against the current, whereas cells up-stream from the center or lateral to it remained unaffected. These data, coupled with the fact that myxamoebae can move tangentially to pre-established streams during aggregation, suggest that the attractive material (termed "acrasin" by Bonner) is produced by both center and streams and that the unaggregated myxamoebae can sense the concentration gradient of acrasin and so determine in which direction to move.

Further study by Bonner (5) revealed that migrating pseudoplasmodia of *D. discoideum* also contain acrasin and can attract unaggregated myxamoeba. Subsequently, Shaffer (48) leached material from single aggregates and pseudoplasmodia and applied it to dispersed amoebae, causing the latter to move towards the source. The agent was found to be extremely labile: all activity was lost by incubation of leached material for fifteen minutes at room temperature. Very recently, we succeeded in obtaining stable preparations of acrasin by leaching large numbers of pseudoplasmodia with water at pH 3.5 (69). The assumption made was that the instability of acrasin is enzymatically contrived by the cells in order to reduce background "noise level" during aggregation and thereby render the concentration gradient more effective. Extraction at pH 3.5 was chosen because it might be in the isoelectric range of the guilty enzyme or at least sufficiently far from the optimal pH to inhibit acrasin destruction. Thus far, we have not had time to test crucially the validity of these speculations and they remain merely attractive possibilities. But in any case the crude acrasin preparations remained indefinitely stable at pH 3.5, although quickly inactivated at pH 7. Passage of the concentrated preparation through cellulose powder columns produced fractions that were also stable at pH 7 (thus providing circumstantial support for the above speculations). When the crude material was run in bar chromatograms on paper, two fractions were obtained, neither of which was biologically active alone, but which in combination could attract myxamoebae. Both the absolute concentrations and ratios of the fractions are critical in this respect. Large amounts of the fractions are now being collected for chemical identification.

Specificity of aggregation.—Raper and Thom (42) have studied the aggregation of interspecific and intergeneric cell mixtures. Some pairs could enter stable communal aggregates, others entered common aggregates but were reshuffled to form separate pseudoplasmodia, and yet others did not mix at all. These findings were extended by Shaffer (48). Leachings from *D. discoideum* and *P. violaceum* were tested on homologous and heterologous myxamoebae. Young pseudoplasmodia of *Polysphondylium* were similarly specific, but at later stages could attract both groups of myxamoebae. The observed specificities were ascribed by Shaffer to differences in the chemotactic agents. *D. discoideum* and young *P. violaceum* were considered to produce only homolo-

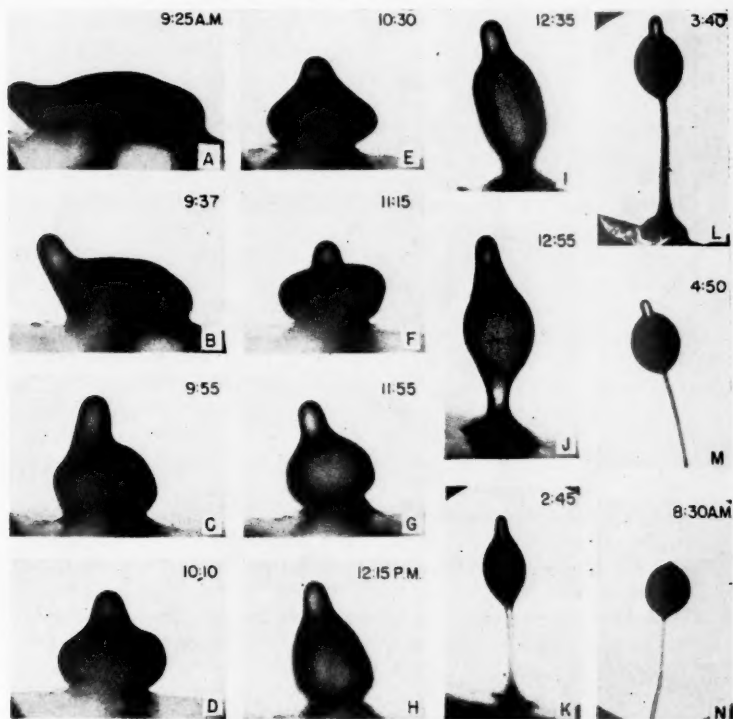


FIG. 3. Serial photographs, in situ, of late pseudoplasmodial migration and fruit construction by *D. discoideum* (45).

gous acrasin; older pseudoplasmodia of *Polysphondylium* could produce both kinds. These interpretations are now complicated by our own findings that acrasin is composed of two substances. The situation should be clarified eventually by chemical identification of the active components.

The influence of population density on center formation.—In 1952, Sussman & Noel (57) published findings that related aggregation, and in particular the number of aggregative centers formed, to population density and the number of cells present. Constant numbers of myxamoeba were dispensed in drops on washed agar over a range of population densities and after incubation the numbers of aggregative centers formed were counted. Figure 4 shows representative curves for *D. discoideum*. The upper curve was obtained with aliquots containing 10^5 cells, the middle curve 5×10^4 cells, and the lower curve with 2.5×10^4 . At very low densities (below eighty cells/mm.²) no centers were formed during 24 hours of incubation. At very high densities, center for-

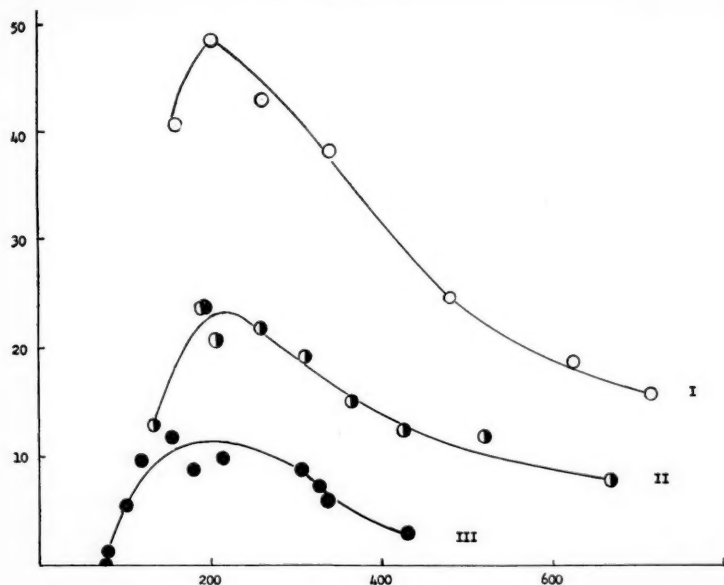


FIG. 4. Relation between center formation and population density in *D. discoideum*. Ordinate: no. centers per aliquot of cells. Abscissa: no. cells/mm². Curve I was obtained with aliquots of 10^5 cells; curve II with 5×10^4 cells; curve III with 2.5×10^4 (57).

mation was depressed, possibly by competition between neighboring center-forming agencies; the resulting centers were larger in size, but fewer in number. Center formation was maximal at 200 cells/mm². The optimal density, as well as the other parameters, is characteristic of the species and strain. The bushy mutant of *D. discoideum* has its optimal density at 350/mm² (64). In another species, *D. purpureum*, the optimal density was found to be 100 cells/mm², while the threshold for aggregation was below 20 (57).

The initiator cell.—From the results given in Figure 4 it is apparent that at the optimal density the number of centers is directly proportional to the number of cells. Thus, for *D. discoideum* wild type the ratio of centers formed to cells present is 1 : 2200. This relationship has since been shown to stem from the fact that each aggregate requires for its formation the presence of a specially endowed cell termed the initiator which, acting singly, induces its neighbors, the responder cells, to begin aggregation.

Replicate population samples containing 1000 or 2000 cells were dispensed in tiny drops on washed agar, at a population density high enough to permit the cells to aggregate. Only a fraction of the samples did so, and the

proportions of samples containing 0, 1, 2, and 3 aggregative centers were in accord with the Poisson series. The data revealed that a very small proportion of the cells in the population could act as initiators (one out of 2200) and that these special cells were distributed at random throughout the samples (57).

A second approach showed that the proportion of cells that can act as initiators is not rigidly determined, but depends upon the nature of the responder cells and the conditions of the experiment. A study was made of the aggregation of cell mixtures containing wild type *D. discoideum* and aggregateless mutants (58). The latter could respond to the aggregative stimulus but could not produce it spontaneously. Drops were dispensed on washed agar containing a constant number of wild type small enough so that alone they were too sparse to aggregate. The presence of aggregateless cells in the drops permitted centers to form. With increasing numbers of the mutant cells, the number of such centers per drop increased until, in the presence of excess aggregateless individuals, a plateau was reached. The plateau value was directly proportional to the number of wild type present in the mixture, indicating that single cells within the wild type component were initiating the aggregates. With one aggregateless stock, one out of 1780 wild type cells could act as initiators; with another, one out of 980. Since that time, H. L. Ennis (unpublished data) has studied about thirty additional aggregateless stocks which display a wide range of sensitivity: some are so sensitive that one out of one hundred wild type can initiate centers among them.

In a subsequent study of wild type *D. discoideum* (68), myxamoebae were harvested at various stages of the growth cycle, including both lag and log phases. After the cells were washed free of bacteria their aggregative performances were examined on washed agar. The population density relationship found for stationary phase cells (Figure 4) was duplicated by all of them. Further, the ratio of initiators to responders remained constant at 1:2200, even in the case of newly germinated spores!

The foregoing studies have cast doubt upon the existence of any rigid qualitative difference between the initiator and responder cells, and indicate rather a spectrum of initiative capacity in the wild type population. Only that proportion of cells whose initiative capacity lies above the threshold value could then act as initiators. The threshold would be determined by the innate sensitivity of the potential responder cells to the aggregative stimulus as well as by how closely the latter could be packed around the putative initiators so as to receive their signal. The growth study further suggests that prior to the stoppage of growth there are no initiator and responder cells *per se*. Instead, the conditions attending growth cessation appear to induce the attainment of initiative capacity. Random physiological differences among the cells may then account for the observed differences in the level of initiative capacity attained.

Genetic control of initiative capacity.—Two mutant strains of *D. discoideum*, isolated after ultraviolet irradiation, have been shown to possess altered initiative capacity (64). One of them, designated fruity (fty-1) is of par-

ticular interest. It was distinguished by its ability to form huge numbers of tiny fruits on growth medium. A study of the mutant's aggregative performance on washed agar revealed that at the optimal density (200 cells/mm.²), one cell out of every 24 could act to initiate an aggregative center. Data obtained from mixtures containing small numbers of the mutant and large numbers of wild type cells showed that most of fruity's enhanced center forming capacity resulted from an absolute increase in the proportion of cells that could act as initiators. This conclusion stemmed from the fact that an average of one out of 60 fruity cells in the mixture induced the wild type responders to form aggregates whereas the wild type initiators themselves are only in the ratio 1:2200. However, since the ratio at 1:24 when the mutant responders were the sole audience, it was concluded that the genetic alteration had also increased the sensitivity of the mutant responder cells to the initiative stimulus.

Environmental factors affecting aggregation.—Hirschberg *et al.* (24, 25, 26) have examined the effects of many enzyme poisons and carcinogenic agents upon aggregation. Unfortunately the test system employed was capable of indicating only gross inhibitory effects. Some of the compounds tested were highly inhibitory; but without viability data, it could not be decided whether the inhibitions represented direct interference with aggregation, or were secondary reflections of generalized cell damage.

Discovery of the dependence of aggregative center formation upon population density and cell number (57) made it possible to use changes in these relationships as criteria of interference with or encouragement of aggregation. Accordingly, an attempt was made to screen a wide variety of physical conditions and chemical additives including vitamins, amino acids, purines and pyrimidines, catabolic intermediates, and redox poisoning agents (15). The system proved remarkably recalcitrant to external influence. A few compounds inhibited aggregation, in some cases accompanied by appreciable cell death, in others not. Adenine and, to a lesser extent, guanine were effective inhibitors of aggregation which did not kill appreciably. Most noteworthy in stimulating aggregation was histidine, which when added to the washed agar increased the sensitivity of the test system enormously. On unsupplemented agar, the density threshold of *D. discoideum* for any center formation at all is 80 cells/mm.²; in the presence of histidine, substantial center formation occurred at densities below 30 cells/mm.². Despite this increase in sensitivity, no change occurred in the initiative capacity of the myxamoebae since the normal distribution of aggregative centers was encountered. The histidine effect was not shown by any other amino acid but among compounds tested that were related to histidine, imidazole acted in like fashion. Studies on histidine uptake by the myxamoebae and on the fate of the intracellular histidine suggested that histidine may be a precursor of either or both components of acrasin; but this interpretation is only one possibility among many.

THE PSEUDOPLASMODIUM

Two general patterns of pseudoplasmodial activity are displayed by the slime molds. In all the species studied thus far, aggregation is followed by a transformation of the conical mound into a vertical, finger-like projection which falls over on its side and becomes recognizable as a pseudoplasmodium. It is at this point that specific differences appear. In *D. mucoroides* and most of the other taxa, the pseudoplasmodium moves over the substratum for a relatively short distance. As it moves forward a rigid, sheathed, parenchymatous stalk emerges from the axial portion of the pseudoplasmodial rear. As soon as the stalk provides a firm base on the substratum, the pseudoplasmodium progresses aerially and enters the culmination stage and fruit construction. In contrast, the pseudoplasmodia of *D. discoideum* migrate far more extensively, covering distances of many centimeters. As a pseudoplasmodium moves it sheds from its outer surface a flexible, ribbon-like cylinder of polysaccharide that looks precisely like a sausage casing. When this extended migratory period draws to a close the pseudoplasmodium changes shape and enters into culmination (Fig. 3). From this point on the performance of *D. discoideum* again becomes homologous with that of the other species. Most of the information about the pseudoplasmodial stage comes from studies of *D. discoideum*.

Pseudoplasmodial organization.—In an elegant study of *D. discoideum* pseudoplasmodia (39), Raper has shown that the anterior tip contains the seat of migratory control. In one experiment anterior segments with a length 10 to 20 per cent of that of the pseudoplasmodium were excised and laid down elsewhere on the agar. In 29 out of 30 operations, the anterior segments continued to migrate over relatively great distances before fruiting. In contrast, the posterior segments moved forward only a few hundred micra to the sites from which the apical tips had been removed and immediately fruited. In the remaining operation, the posterior segment developed a new apical tip and resumed migration. A second experimental series revealed that the removal of as little as one per cent of the pseudoplasmodial length at its anterior end could destroy the migratory capacity of a significant fraction of posterior segments. The argument that the act of fragmentation had itself injured the posterior and prevented further migration was answered by cutting pseudoplasmodia and leaving the segments otherwise undisturbed. The apposite fragments rapidly coalesced and continued to migrate.

Raper also stained the pseudoplasmodia vitally by allowing the vegetative myxamoebae to feed upon *Serratia marcescens* prior to aggregation. *D. discoideum* can neither digest the red pigment nor eliminate it. Apical tips from the red pseudoplasmodia were grafted onto unpigmented posterior segments and vice versa. The two areas invariably remained distinct indicating that the relative positions of cells in the pseudoplasmodium are rigidly fixed. Later studies by Bonner (7) confirmed and extended Raper's findings. Attempts to make various kinds of pseudoplasmodial grafts demonstrated the existence of

a high degree of geometric specificity. For example, apical tips could be grafted only to posteriors and further the original orientations had to be maintained in so doing. Apex-apex and posterior-posterior grafts were ineffective.

Production of acrasin by the pseudoplasmodium.—Bonner (5) placed pseudoplasmodial segments in contact with myxamoebae which were themselves ready to aggregate spontaneously. The latter immediately became oriented to and moved toward the former. Comparison of the areas of influence of such segments made it possible to infer that the apical 10 per cent of the pseudoplasmodium produced about 50 per cent of the acrasin.

Sensitivity to external conditions.—Raper (40) reported that the pseudoplasmodium of *D. discoideum* would migrate toward incident light and also in the direction of increasing temperature. Bonner *et al.* (8) confirmed and extended these observations. A quantitative examination of the light response showed all tested wave lengths in the visible range to be equally effective. A heat gradient of 0.05°C./cm. was found to be the threshold for attraction.

The migration time of pseudoplasmodia on agar is limited by the presence of electrolytes and nonelectrolytes, the former being more effective than the latter (54). In the absence of any additives (plain agar) a mean migration time of 10 days was observed, some pseudoplasmodia migrating as long as 20 days before fruiting. Increasing concentrations of solutes cut this migration period progressively to zero. In our experience with washed agar, migration encompasses a span of no more than a few hours, suggesting that the plain agar must itself contain a contaminant that stimulates migration.

Rate of migration.—Bonner *et al.* have determined that the rate of migration is proportional to the pseudoplasmodial volume (11). By analogy with experiments of Tyler on Echinoderm embryos (70), this can be taken to indicate that the motive force is supplied by all of the component cells and not merely by the superficial layer. The lowest observed rate was 0.25 mm./hour and the largest, 2.0 (3).

THE FRUITING BODY

Culmination.—Many workers have described the culminative process. The most notable modern study is by Raper & Fennell (45). The following description of fruiting in *D. discoideum*, quoted from a previous publication (66), is based primarily upon the work of these authors.

At the beginning of the process the pseudoplasmodium rounds up and the anterior tip surmounts the whole to become an apical papilla. A delicate hyaline membrane, the sheath initial, appears at this time underneath the apex and the entire structure then flattens into a crown like shape. Although no morphological distinctions between the cells can be made at this time, those within the membrane are destined to form the lower part of the stalk. When as a result of the flattening, the sheath initial comes into contact with the substratum the mass of myxamoebae above and outside of it begins to rise. The cells at the apex invaginate and ultimately become the upper stalk, while the others, prespore cells, move up and around them. Concurrently, the bottom-most myxamoebae are constricted off from the rest by a creasing of the pseudoplas-

modial slime envelope, thus producing the basal disk. As the mass of myxamoebae rises and the stalk increases in height, the sheath is itself made thicker and higher owing to the extracellular deposition of cellulose by the stalk cells. The upward motion of the myxamoebae appears to be due partly to the accumulative pseudopodial motions and partly to the progressive swelling and vacuolation of the stalk cells. At this time the cells at the upper and outer portion of the rising mass are transformed into spores and the transformation spreads inward and downward until all the cells that have not entered stalk have become spores.

A modification of this basic pattern is seen in the genus *Polysphondylium*. Many spore masses, each with a short stalk, branch in spiral whorls from the main stalk.

The origin of component tissues.—The available data clearly indicate that whether a given cell is to become a spore, stalk, or basal disk cell depends upon its position in the pseudoplasmodium, which in turn is related to the time at which it entered the aggregate. Raper (39) grafted pigmented anterior pseudoplasmodial segments to colorless posteriors and, as mentioned previously, the two areas remained distinct during migration. Migration completed, the pigmented apex invaginated through the colorless posterior and gave rise to a colored lower stalk while the spore mass, upper stalk, and the basal disk were not colored. The extent of the pigmented portion depended upon the relative sizes of the grafted segments. Bonner (2, 7) marked parts of the radiate aggregate pattern with a vital dye and followed the marked areas through the developmental cycle. The myxamoebae that first entered the aggregate became the apical tip of the pseudoplasmodium and eventually the lower stalk of the fruit. Myxamoebae that entered the aggregate at a later time occupied progressively more posterior parts of the pseudoplasmodium, and eventually formed the upper stalk and spore mass of the fruit. Those that entered the aggregate last formed the rear of the pseudoplasmodium and the basal disk of the fruit.

Some information is available concerning the persistence of this geometrically induced differentiation. Sussman found (56) that clones produced by stalk cells showed no developmental aberrations and could supply all the cell types required for normal fruit construction. Thus the differentiated state cannot persist through the cycle of the vegetative growth. More pertinent, Raper (39) and Bonner (7) showed that when pseudoplasmodia were segmented and the posterior portions stopped migrating, they constructed normal fruits containing the usual proportion of stalk cells and spores. In contrast, the anterior segments that migrated a short distance after the operation formed fruits that were practically all stalk and no spore mass. When the anterior portions could migrate over greater distances the resulting fruits became progressively more normal in appearance and approached the usual proportion of stalk cells and spores. Thus under these conditions the differentiation persists for a short time, but it is not maintained, even in the absence of appreciable growth. It remains possible that conditions might be found in which the differences could persist. Nevertheless, since differentia-

tion does involve large numbers of cells and appears to be easily reversible in the absence of growth, the events that precede the expression of spore, stalk, or basal disk cell potencies must not include changes in genic constitution. It appears more likely that the fate of a cell (i.e. whether it is to become spore, stalk, or basal disk) is determined by cellular interactions within the pseudoplasmodium that result in nonpersistent cytoplasmic alterations. Results obtained with morphogenetically deficient mutants (61), to be discussed in another section of this review, suggest that at least some of these interactions proceed via direct cell-cell contact and not by exchange of diffusible intermediates.

Profound differences have also been observed in the metabolic activities of prespore and prestalk cells both in the pseudoplasmodium and during culmination. The prestalk cells were found to lose appreciable amounts of protein nitrogen, quantitatively recovered as ammonia (22). The conclusion that protein was employed as an energy source is consistent with our own nutritional data (62). It is interesting that the activity of the prespore cells in this respect was negligible but that of the prestalk cells, very great. The prestalk cells were found (13, 23) to be extremely active in other ways (i.e. polysaccharide synthesis, alkaline phosphatase activity) while the prespore cells appeared to conserve energy and materials during preparation for dormancy.

Regulation of the gross proportions and histological composition of the fruit—Regulation is a term originated by embryologists, to describe the ability of a morphogenetic system to exert rigid control over the relative amounts of component tissues and the gross proportions of the finished product. Further it is a measure of the ability of such a system to turn out a normal product regardless of the absolute number of cells taking part. In terms of the latter criterion the slime molds possess a truly spectacular morphogenetic talent.

Because the size of the aggregate (and that of the fruit) depends upon the population density (57) fruiting bodies can vary widely in size. In *D. discoideum* wild type, fruits have been found (41) to range from about 5 mm. in height (containing many tens of thousands of cells) to less than 1 mm. in height (containing about 500 cells). Yet despite the disparity in numbers, such fruits were perfectly regulated. The criteria employed for these comparisons included the following ratios: spore mass diameter to stalk height; number of component cells to stalk height; dimensions of stalk cells to dimensions of spores; number of stalk cells to number of spores (6, 12, 41). The fruity mutant of *D. discoideum* at its optimal population density was found to produce an average of one aggregate among every 24 cells present (24). The resulting fruits were correspondingly tiny, some, in fact, being composed of 12 cells or less; the fruit shown in Figure 5 is an example. Such fruits showed precisely the same spore, stalk, basal disk cell ratios and gross proportions as did their wild type counterparts. Thus, the scale over which the system can regulate efficiency now extends from assemblies containing tens of thousands of cells to those containing less than a dozen individuals.

This feature of slime mold development is particularly important because, as previously discussed, the fate of a given cell appears to be governed by cellular interactions operating during and after the pseudoplasmodial stage. Thus the proportions of the component tissues in the fruit must be a function of the matrix of interactions. The fact that the system can regulate itself so effectively even in assemblies containing 12 cells or less indicates that these interactions do not require the combined efforts of large numbers of individuals. In the past, the experimental dissection of cell interactions in morphogenetic systems has been impossible because of their complexity and of the huge numbers of cells involved. As seen, this impedance does not exist in the case of the slime molds. The fruity mutant should provide the means for an incisive attack upon the problem of these cell interactions which indeed are the basic feature of morphogenetic systems (67).

GENETICS AND CYTOGENETICS

MUTANT STRAINS

In recent years, a large part of our own efforts have been spent in collecting and examining mutant strains of *Dictyostelium*, particularly those displaying aberrant morphogenetic patterns (59). The interest in these forms springs not so much from their purely genetic aspect as from the insights they can provide into the genetic and physiological processes attendant upon morphogenesis.

At present, some 92 mutant stocks of *D. discoideum*, *D. mucoroides*, and *D. purpureum* have been isolated after ultraviolet irradiation of spores and myxamoebae (Table I). Illustrations and detailed descriptions are available elsewhere (59, 61, 64). The independent isolates mentioned for each category in Table I are not necessarily identical and, in fact, frequently show minor phenomic differences amongst themselves.

The developmental mutants are of two kinds. In one group, morphogenesis is complete in the sense that terminal fruits are formed, but deviations from the normal sequence of events lead to grossly aberrant structures. In some cases, the aberration is simply one of size, number of fruits (dwarf, fruity, long-stemmed), or both. In others, the stalks become tortuously curled, thick with slime, and all demarcation between spore mass and stalk is lost (curly, glassy). In still others, branching and multiple stalk formation occur (bushy, forked). Minor changes such as in pigment formation are also encountered (brown, albino). It should be noted that many of these deviations, as well as others not yet encountered after mutagenic treatments, are apparent in natural isolates, particularly of *D. mucoroides*.

The morphogenetic capacities of fruity, dwarf, and bushy have been examined in detail (64). As previously described, the phenotypes of the first two can be satisfactorily accounted for by the observed increases in the proportion of initiator cells and in the enhanced sensitivity of the responders.

TABLE I

LIST OF MUTANTS ISOLATED AFTER ULTRAVIOLET TREATMENT AND RETAINED FOR STUDY

Species	Type of phenomic deviation	Name of mutant	No. of independent isolates
<i>D. discoideum</i>	Pigmentation of spore mass	Albino	1
		Brown	1
	Nutritional	Auxotroph	3
	Morphogenetically deviant	Bushy	6
		Dwarf	2
		Fruity	4
	Morphogenetically deficient	Fruitless	11
		Aggregateless	44
	Nutritional	Auxotroph	2
		Morphogenetically deviant	1
<i>D. mucoroides</i>	Morphogenetically deviant	Dwarf	1
		Forked	1
	Morphogenetically deficient	Curly	2
		Glassy	1
		Long stemmed	1
	Morphogenetically deficient	Fruitless	7
		Aggregateless	1
	Morphogenetically deviant	Bushy	1
		Morphogenetically deficient	3
	Morphogenetically deficient	Aggregateless	3

The bushy character is associated with a change in the population density relationship during aggregation but it is not clear what this has to do with the structural aberrations that ultimately appear.

Cohen (19) succeeded in producing phenocopies of many of these mutant types by incubation of wild type *D. discoideum* under atmospheres contaminated with relatively large amounts of ammonia or carbon dioxide. No persistent effects of any kind were observed when such aberrant fruits were subcultured in the absence of these gases.

The second class of mutants has been termed morphogenetically deficient (61). This includes stocks whose plaques on growth medium do not display complete developmental competence. Some plaques are aggregateless so that after the cessation of growth the myxamoebae remain in a separated

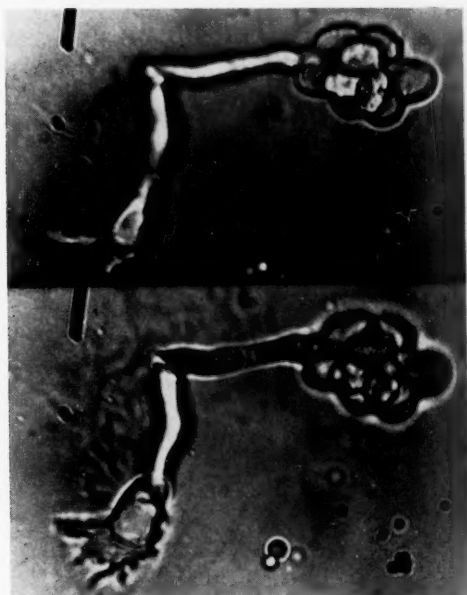


FIG. 5. Views in two focal planes of a fruit formed by the mutant, Fty-1. The structure is composed of 9 spores, 2 stalk cells and 1 basal disk cell. Magnification $\times 1760$ (64).

condition. Some are fruitless so that the myxamoebae aggregate but develop no further, or not much further, depending on the individual strain. Such stocks can be additionally subdivided by their developmental capacities on washed agar. Washed myxamoeboid suspensions of some aggregateless and fruitless strains when dispensed on washed agar reflect precisely their corresponding capacities on growth medium. Others can develop further, however, even to the production of terminal fruits with viable spores. The latter when subcultured produced plaques displaying the original deficient phenotype. Obviously their morphogenetic potentials are curbed by something in the growth medium or the bacterial associate.

SYNERGISTIC AND ANTAGONISTIC INTERACTIONS AMONG MORPHOGENETICALLY DEFICIENT MUTANTS

Strain Fr-1 is a fruitless stock whose morphogenetic capacity is identical on growth medium and on washed agar. The myxamoebae enter into flat, round, loose aggregates that remain stationary and develop no further. Agg-53 is completely aggregateless under both conditions. When suspensions of the two strains were mixed and dispensed on washed agar, a synergistic reac-

tion occurred such that the two together could develop much further than when alone (60, 61). The synergistic morphogenesis took two forms. In some cases, perfectly normal conical aggregates were produced which did not migrate appreciably, but which were transformed into mature normal fruits with viable spores. In other cases only flat aggregates appeared, but these, unlike the flat aggregates formed by Fr-1 alone, could migrate extensively over the agar surface. No further development occurred in these "pseudopseudoplasmodia." Both the type and intensity of synergism were found to depend on the cell ratio of the two mutant strains in the mixture. When Fr-1 was greatly in excess no fruits were formed, but only flat migrating aggregates. When Fr-1 and Agg-53 were present in approximately equal numbers, both flat migrating aggregates and mature fruits appeared. When Agg-53 was in excess only fruits were observed.

With other pairs of deficient mutants different types of synergistic activity have been observed. Of particular interest were pairs which included Agg-204, a stock isolated not from wild type *D. discoideum*, but from the bushy variant, Bu-1. When Agg-204 predominated in synergistic mixtures the fruits were typically bushy in appearance and not wild type. Thus, the genetic potential for the bushy type of morphogenesis is carried in Agg-204 even though the subsequent genetic alteration to an aggregateless state does not permit the phenotypic expression of that potential, except under synergistic circumstances.

A number of distinct synergistic response patterns was observed when seven fruitless and six aggregateless strains were mixed in all possible paired combinations (Table II). Among the aggregateless stocks, three independent patterns were apparent. Thus, Agg-59 and Agg-204 responded synergistically with Fr-1, Fr-2, and Fr-3, but with no other stock. Agg-53, Agg-53A, and Agg-57 responded with Fr-1 and Fr-3, but with no other. Agg-206 responded only with Fr-3. Amongst the fruitless stocks, seven different response patterns were observed. This would imply that at least three distinct metabolic alterations can lead to the aggregateless condition, while at least seven lead to fruitlessness. Note that the results are not the simplest inherent in this kind of analysis, namely that all strains with different deficiencies should synergize when mixed, and only those with the same should not. This complication does not invalidate the conclusions reached, but means merely that the system is not sensitive enough to reveal all the possible synergistic relations. Surprisingly enough, no two aggregateless stocks have been found to synergize, even though study has recently been extended to include 34 additional aggregateless mutants of *D. discoideum*.

One interesting case of antagonistic interaction was uncovered (61). Agg-208 is a stock that produces aggregateless plaques on growth medium but complete fruits with viable spores on washed agar. Agg-206 is aggregateless in both circumstances. In mixtures of the two, the ability of the former to fruit was completely repressed, even when the latter was present in a cell ratio of as little as 10:1. The antagonism was extremely specific and the in-

TABLE II

COMPARATIVE RESPONSE PATTERNS OF SEVEN FRUITLESS AND SIX AGGREGATELESS
MUTANTS OF *D. DISCOIDEUM*. X=SYNERGISTIC COMBINATIONS.
—=NONSYNERGISTIC COMBINATIONS

	Fr-1	Fr-2	Fr-3	Fr-4	Fr-5	Fr-6	Fr-7	53	53-A	57	59	204	206
Fr-1		—	—	X	—	X	X	X	X	X	X	X	—
Fr-2	—		—	—	—	—	—	—	—	—	X	X	—
Fr-3	—	—		—	X	X	X	X	X	X	X	X	X
Fr-4	X	—	—		X	—	X	—	—	—	—	—	—
Fr-5	—	—	X	X		—	—	—	—	—	—	—	—
Fr-6	X	—	X	—	—		—	—	—	—	—	—	—
Fr-7	X	—	X	X	—	—		—	—	—	—	—	—
Agg-53	X	—	X	—	—	—	—		—	—	—	—	—
53-A	X	—	X	—	—	—	—	—		—	—	—	—
57	X	—	X	—	—	—	—	—	—		—	—	—
59	X	X	X	—	—	—	—	—	—	—		—	—
204	X	X	X	—	—	—	—	—	—	—	—		—
206	—	—	X	—	—	—	—	—	—	—	—	—	

hibitory activity of Agg-206 could be dissociated from viability of the cells by freezing and thawing.

MECHANISM OF SYNERGISTIC DEVELOPMENT

By analogy with the syntrophic relations that obtain among auxotrophic mutants of *N. crassa* and *E. coli*, the simplest explanation of synergistic development seemed to be that the deficient strains were exchanging diffusible intermediates. When preliminary attempts to demonstrate exchange by the use of extracts and tenderly killed myxamoebae proved fruitless, a more rigorous test was devised (63, 65).

Very thin agar membranes, as little as thirty microns in width, were prepared by dipping flanged, stainless steel cylinders into molten washed agar. The agar was allowed to set, and the resulting membranes were incubated in a water-saturated atmosphere to prevent desiccation. Synergistic pairs were then deposited on opposite sides of a membrane. Although a wide range of cell ratios was employed, cells separated by the agar never showed synergistic development, although when strains were mixed on one side of the membrane, the expected synergism was observed. The significance of this result was enhanced by the demonstration that acrasin, a complement of highly labile diffusible substances, could penetrate membranes as much as 200 micra thick. Thus, when wild type myxamoebae opposed each other, the ensuing aggregates on either side of the membrane coincided precisely, center for center, and cell stream for cell stream. In passing, it must be mentioned that the agar membranes should prove useful and convenient for many situations where populational interactions must be studied on a solid substratum. They

are easy to prepare and maintain free from contamination. Holes in the membrane are never encountered as long as those with air bubbles are initially discarded.

A second possibility is that the deficient cells can produce genetically competent zygotes, heterokaryons, or meiotic products by syngamy. Spores from the synergistic fruits were plated and the resulting clones examined for evidence of recombination. In no case were these observed (61). Only the parental types emerged. It is interesting that the proportions of myxamoebae in the original mixture dictated the proportion of parental types among the spores. R. R. Sussman (unpublished data) has examined the clones obtained from mixed populations in the intermediate stages of synergistic development with the same results. This rules out the possibility of anything but extremely transient heterokaryosis to explain the synergism.

Because some of the details of synergistic fruiting compel one to think that some sort of material exchange must occur between the deficient partners (61), we have examined the possibility that the cells exchange nondiffusible substances by direct contact, perhaps by cytoplasmic exchange. The latter mechanism is operationally identical with the supposition of transient heterokaryosis. We are now engaged in an attempt to validate the possibility. Other types of direct cell-cell contact interactions in development can be envisioned. However, we think the above is the most likely and at present amenable to direct test.

THE POSSIBILITY OF SEXUALITY

There is little evidence at present to support the occurrence of sexuality in the cellular slime molds. One modern attempt to provide cytological support has accumulated only inconclusive data (72, 73). Our own search for signs of genetic recombination has thus far produced nothing.

On the basis of earlier speculations by Skupienski (53) concerning *D. mucoroides*, Wilson has proposed a scheme for *D. discoideum* in which syngamy and meiosis are mandatory accompaniments to the fruiting process (72, 73). According to this worker, syngamy must precede aggregation, and only zygotes can enter the aggregate which is initiated by the first zygotes formed. Meiosis commences in the aggregate soon after the zygotes enter, and all cells are once again returned to the haplophase. Near the close of the developmental sequence, when the construction of the fruit is almost completed, a wave of mitotic divisions is said to occur immediately prior to sporogenesis so that each zygote ultimately produces sixteen spores.

Cytological evidence for this scheme does not seem to be compelling. According to Wilson, syngamy has been observed only rarely and then only at the outer fringes of already formed aggregates. Thus, the suppositions that zygotes initiate the aggregate and that syngamy is universal are unsupported. Further, the act of fusion is described by this worker as the pseudopodial envelopment of one myxamoeba by another. It is important to realize that the cells at this time were deprived of food and could easily have been engaged

in eating rather than mating. Cannibalism is well known among the Sarcodina. Since the photograph of cells in the process of fusion (73) shows two completely discrete nuclei with no indication of synkaryosis, the data do not negate the possibility of cannibalism.

The evidence for meiosis consists of three photographs purportedly showing late prophase, anaphase I, prometaphase I, and prometaphase II. One cannot determine from these photographs whether the chromosomal figures were meiotic or mitotic, certainly not from the relative sizes of the chromosomes. It is interesting to note here that Bonner & Frascella (9), in studying the same post-aggregative divisions, took them to be mitotic rather than meiotic. In addition, these workers, by determining the incidence of such figures, revealed that two waves of division occur. One took place during late aggregation among the first cells to enter the aggregate (pre-stalk cells); the second wave to be found in the newly formed plasmodium occurring among the remainder of the cells (pre-spore and basal disk cells). During these periods, the incidence of division figures was no more than 2 to 3 per cent. No divisions were observed immediately prior to aggregation in agreement with our own data (58) obtained from viable and direct cell counts. Wilson cautions that his estimate of sixteen spores originating from each zygote is only approximate, being based on the relative sizes of the pre-spore cells and the terminal spores.

Two rather powerful arguments can be raised against the proposed scheme. The first refers to the supposition that aggregates are initiated not by special initiator cells present in small proportions in the population, but rather by the first zygotes formed. Were this so, the distribution of centers among small replicate populations at high density could not be in accord with the Poisson expression (57). Instead, since according to Wilson zygotis is universal, every such sample should have produced at least one center. The same inconsistency is apparent from the results obtained by mixing wild type and aggregateless cells (58).

Second, according to this scheme, any population that can aggregate must be able to mate and, indeed, any cells entering the aggregate must be zygotes. This would dictate that mixed aggregates between genetically marked stocks should provide an appreciable number of recombinant individuals. Mixed aggregates have been made under a variety of conditions using a large number of mutants listed in Table I (R. R. Sussman, unpublished data). The stocks, known to be able to enter into mixed fruits, have been grown together and also grown separately and incubated together after washing. Washed agar, SM medium, and the medium used by Wilson have been employed. As many as 10,000 spores from a single mixed fruiting have been grown clonally and the plaques examined for evidence of recombination. Mixed aggregates and pseudoplasmodia have been broken up and also examined in this way. Mutants of two species, *D. discoideum* and *D. mucoroides*, were employed. No recombinant clone has ever been isolated. The possibility does exist that all the marked stocks are infertile, although the variety of

markers used makes it a remote one. Even if this were so, however, it would still mean that sex is not a mandatory accompaniment to fruiting. In the meantime, a further search is being carried out for evidence of recombination among more than 50 independent natural isolates of *D. mucoroides*. The possibility entertained is that heterothallism exists or that a sexual cycle may operate apart from the sequence of fruit construction. Unfortunately, however, a search for genetic recombination without cytological guideposts is beset with risks. The data of Lederberg (29, 30) indicate that in the celibate era of bacteriology the probability that he would find two fertile strains of *E. coli* was approximately 1:50.

PHYLOGENY AND SYSTEMATICS

PHYLOGENY

The perplexing problems presented to zoological and botanical systematists by the slime molds are exemplified by the fact that the group has a dual classification. The botanical system grants it the divisional rank of Myxomycophyta; the zoological allots it the order Mycetozoa. This is the predictable fate of forms which on the one hand can exist as amoebae, morphologically and ecologically similar to the Sarcodina, and on the other, can construct stalked or sessile fruiting bodies that simulate true fungal morphology to an extraordinary degree. The possibility that both relationships have been validly drawn and that the slime molds have indeed served as an evolutionary bridge between protozoa and protophyta has not yet been seriously investigated, but offers an exciting opportunity for study.

I have chosen to employ the botanical classification for no other reason save that it has been used more frequently in the past. The taxon Myxomycophyta comprises four classes, including two of true slime molds, Myxomycetae and Plasmodiophoreae, whose spores germinate into flagellate swarm cells, which in turn become huge, multinucleate, amoeboid plasmodia; the Labyrinthulae, whose cells are joined by cytoplasmic strand to form a net plasmodium; and lastly, the cellular slime molds or Acrasieae. The relationships among these classes (excepting those between the first two) are certainly not obvious. Their life cycles do not display strict homologies and the main though unstated reasons for bringing them together seem to be convenience and historical precedent. The Acrasieae then have no indisputable ties with any other group, though many likely but unproven ones exist. A rigorous comparison of nutritional patterns and intermediary metabolism is not currently possible, nor has a study yet been made of serotypical relationships within this group or between it and other groups.

One other phylogenetic aspect of the Acrasieae is noteworthy. It has to do with the evolution of stable multicellularity, the specific steps through which protista became metazoa and metaphyta. In a sense, the latter forms might also be called "unstable" protista if one remembers the fleeting period during which gametic individuals approach and fuse into a single-celled zygote. Thus,

the evolution of multicellularity can be considered to be a passage from the protistal extreme in which unicellularity occupies the entire developmental cycle to the opposite extreme where it is reduced to a transient phase. The intermediate forms in this succession might be expected to resemble the Acrasieae in that the protistal and multicellular conditions would both play prominent roles in the developmental sequence. From this point of view the Acrasieae assume great biological importance, for they may well have been in the mainstream of that evolutionary transition.

TAXONOMY OF THE ACRASIEAE

The basis for the modern classification of the cellular slime molds was laid down by Olive (31) in a paper that remains an exquisite example of classical observational microbiology. Modifications and extensions have since been supplied by study of natural isolates (34, 35, 43, 49, 50, 51), and of ultraviolet induced mutants of known species (59). According to Olive's system, the class contains one order, the Acrasiales, and three families distinguished by progressive complication of the morphogenetic sequence. They are:

Family Sappiniaceae.—The myxamoebae do not construct fruiting bodies, but form pseudospores directly. (Pseudospores are small rounded cells with apparently rigid walls but not greatly thickened as in true spores. Size and shape also differ from those of true spores.) Frequently, the pseudospores are collected in random clumps. One genus, *Sappina*, with two species. A careful reexamination of this family is imperative. Most of the properties described by Olive (31, 32) correspond in detail with those of the genus *Hartmanella* (Sarcodina), and in particular with the recently isolated species, *H. astronyxis* (46). For example, Olive's drawing of a pseudospore is almost identical with Ray's photographs of encysted *Hartmanella*. Because the *Sappinia* were not growing under optimal conditions at the time they were studied by Olive, it is not possible to make a definitive judgment concerning whether *Sappina pedata* (21) and *Hartmanella astronyxis* are one and the same or whether the Sappiniaceae are in fact sufficiently different from the Hartmannellidae to warrant grouping them with the Acrasieae.

Family Guttulinaceae.—The myxamoebae aggregate and then, depending on the genus, either remain as a rounded or a conical mass of cells or construct a crude, amorphous fruit with a short, thick stalk. Pseudospores are found in the former type, true spores in the latter. Two genera (*Guttulinopsis* and *Guttulina*) with seven species.

Two considerations indicate that this group might also require taxonomic revision. Raper (36, 37), Singh (50), and Cohen (19) have shown that the growth of *Dictyostelium* under sub-optimal conditions can cause aberrant development to the point where the fruits markedly resemble those described for the Guttulinaceae. Further, as previously described, a large number of morphogenetically deficient mutants of *Dictyostelium* (59, 61) can only produce conical cell aggregates or immature fruits; others are similarly deficient on growth medium, but can produce normal fruits on washed agar. Since in

1902, Olive knew little about slime mold nutrition and nothing of its genetics, it is conceivable that the Guttulinaceae described by him were merely unhappy Dictyosteliaceae or mutants thereof.

Family Dictyosteliaceae.—The myxamoebae aggregate, form pseudoplasmodia and ultimately fruiting bodies. The sori contain true spores and the stalks are parenchymatous and sheathed with cellulose. Five genera and seventeen species.

Genus *Acrasis* van Tieghem, 1880 (71): Concatenate spores, terminating a thin stalk, consisting of singly tiered cells. (One species described by van Tieghem but never reisolated.)

Genus *Dictyostelium* Brefeld, 1869 (16): Spherical or subglobose sori atop simple stalks comprising the following species:

1. *D. discoideum* Raper, 1935 (35): Yellow sori; straight tapered stalks with differentiated basal disk; pseudoplasmodia migrate over long distances.
2. *D. mucoroides* Brefeld, 1869 (16): Milk-white or cream sori with flexuous stalks. Highly pleomorphic. (Two of the species cited by Olive (31, 32), *D. sphaerocephalum* and *D. brevicaulis*, and one designated *D. giganteum* by Singh (50) have been considered (44) part of the *D. mucoroides* complex. From his own experience with natural isolates and with mutants of *D. mucoroides*, the writer agrees with this opinion.)
3. *D. purpureum* Olive, 1901 (31): Large purple sori on long and flexuous stalks.
4. *D. lacteum* van Tieghem, 1880 (71): Milk-white sori containing exceedingly small spores atop thin stalks composed of singly tiered cells.
5. *D. aureum* Olive, 1901 (31) and *D. roseum* van Tieghem, 1880 (71): Distinguished primarily by the yellow-gold and rose pigments of the respective sori. (We have isolated mutants of other species possessing colorless, deep orange, and red-brown sori. Clearly, sorus pigment alone is not a sufficient criterion for species designation. Neither of these forms has been reisolated.)
6. *D. minutum* Raper, 1941 (43): Tiny white sori atop thin, short stalks, more or less clustered and frequently branched. Occasional strains produce "macrocyts" which may represent abortive fruits.

Genus *Polysphondylium* Brefeld, 1884 (17): Spherical sori born on branched stalks. The branches are in whorls about the main axis. Three species have been designated primarily on the basis of sorus pigment, but also other criteria. The species: (a) *P. violaceum* Brefeld, 1884 (17) (b) *P. pallidum* Olive, 1901 (31) (c) *P. album* Olive, 1901 (31). These are extremely ubiquitous forms in the soil, rivaling *Dictyostelium mucoroides* in this respect.

Genus *Coenonia* van Tieghem, 1880 (71): A tapered stalk with basal disk expanding at the apex into a cupule with finely toothed edges, each peripheral stalk cell bearing a tooth or papilla at the exposed side. The

sorus sits inside this cup. One species, *C. denticulata*. This remarkable form has not been seen since its original description by van Tieghem. It should be of interest to all microbiologists to learn that a prominent contemporary investigator has offered a bottle of the finest Scotch whiskey as a prize for the first reisolation of this species. The name of the Donor and rules of the contest will be supplied upon written request.

Very recently two new organisms have been isolated by K. B. Raper. One has been given the name *Dictyostelium polycephalum* and the other has been placed in a new genus, *Acytostelium* (*A. leptosomum*). Published accounts of these organisms had not appeared at this writing.

ECOLOGY AND METHODS OF ISOLATION

Brefeld (16) isolated the first of the Acrasieae, *Dictyostelium mucoroides*, from dung, and subsequent workers in collecting other species acted upon the assumption that they were coprophilic. Then the Krzemieniewskis (27) reported that in Poland *D. mucoroides* could be isolated from cultivated soil and *Polysphondylium violaceum* and *P. pallidum* from forest substrate. Thereafter, Acrasieae were encountered (20, 34, 44) in a wide variety of soil habitats. They are also present profusely in soil around wild and cultivated herbaceous plants (M. Sussman, unpublished data), and in turf (50, 51). It may therefore be concluded that the normal habitat of the Acrasieae is soil, and specifically niches containing large numbers of soil bacteria. It is conceivable that the myxamoebae may also thrive in pond water given a low bacterial density, but no systematic search has been made thus far.

A complete and accurate census of the Acrasieae has not yet been taken. The main technical hindrance has been the inability to grow the organisms clonally so as to count viable individuals present in natural samples. Clonal growth can now be achieved (55, 56) and it should be possible to spread homogenized samples with perhaps a variety of bacterial associates on dilute nutrient or plain agar media and obtain an accurate picture of the incidence of these forms. The frequency of isolations from natural samples does give at least an indication of the ubiquity of the Acrasieae, and several workers have contributed pertinent data. Raper (44) collected soil and leaf mold from deciduous forests in Virginia, Illinois, and Indiana. Thirty-seven out of forty-one samples contained at least one species of Acrasieae. *D. mucoroides* was isolated 22 times; *D. minutum* 13; *D. purpureum* 7; *D. lacteum* 5; *D. discoideum* 3; *P. violaceum* 17; *P. pallidum* 11. Cohen (20) reported on collections made from a forest floor in Georgia and confirmed the relative frequencies shown above. From 52 soil samples taken from areas supporting herbaceous plants, trees and grass in the environs of the Northwestern campus, the Cook County Forest Preserve and the Winnetka Golf Course, the writer recently collected 24 isolates of the *D. mucoroides* complex (including *P. brevicaulis* and *P. giganteum*), 16 of *P. violaceum* and *P. pallidum*, one of *D. purpureum*, and another that may possibly be the equivalent of Olive's *D. aureum*. Surprisingly, Singh (50, 51) found only *D. mucoroides* and *D. giganteum* in British cultivated soils. However, comparisons are meaningless since no details are

available in any of the published works regarding the quantities of soil assayed or the numbers of plates used. Further, Raper observed (44) that the Acrasieae were particularly abundant at the beginning and end of the growing season. It is possible that Singh collected at an off period.

A controlled study of the relation between myxamoebae and bacteria in soil has been made by Singh (51). Measured amounts of sterile soil were inoculated with spores of *D. mucoroides* and of *D. giganteum*, together with a soil bacterium (strain 4002, otherwise unidentified). The soil was sampled at intervals and viable counts were made of the added bacteria. After about 10 days the bacterial count in the absence of slime molds was 4.5×10^8 per gram soil; in the presence of *D. giganteum* about 1.5×10^8 , and with *D. mucoroides* 7×10^7 . Thereafter the first two counts slowly diminished, the third slowly rose. Non-sterile soil also permitted abundant myxamoeboid growth. The results indicate that the Acrasieae play a major role in soil economy by helping to control the bacterial population level. Presumably they in turn serve as prey for larger protozoa and metazoa.

Relatively little attention has been paid to the morphogenetic process in the natural habitat. Singh (51) found that *D. mucoroides* and *D. giganteum* could spread through and fruit normally in soil if the water content were high enough. Kubiena (see 51), in a detailed micropedological study made by direct observation of various soils, photographed fruits that are unmistakably those of *Dictyostelium*. Thus we can at least be confident that the fruiting process is carried out actively in soil.

Almost every investigator has evolved his own recipe for isolation. The techniques are summarized as follows: (a) Loopfuls of bacteria are spread in small one-inch diameter circles on plain agar. A few crumbs of soil or drops of soil suspension are added at the center of each circle (51). (b) Suspensions of soil, leaf mold, or other rotted vegetation are streaked on dilute hay infusion agar. Growth of myxamoebae depends on development of bacteria present in sample (44). (c) Cohen (20) uses mannitol agar in which he imbeds four autoclaved rabbit dung balls at equidistant intervals. He then pours a soil suspension onto the plate and decants after an hour. (d) A few drops of a culture of *E. coli* or *A. aerogenes* are spread over the entire surfaces of plain agar plates. Pinches of soil are then ground between thumb and forefinger, allowing a fine dust to settle over each plate. Alternatively, suspensions of soil are made and a few drops of supernatant fluid spread on the plates with a drop of bacterial culture. Plaques of Acrasieae appear in the bacterial growth area after a few days of incubation (Shaffer, Bonner & Sussman, unpublished data).

Records of isolations indicate that there is probably little to choose among these methods. All provide large numbers of isolates with comparatively little effort.

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REVIEW OF THE MICROBIOLOGICAL AND IMMUNOLOGICAL LITERATURE PUBLISHED IN 1955 IN THE U.S.S.R.¹

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In writing this review the first aim of the author was to be objective and to avoid any ideological inference; he was to inform, not to discuss. Moreover, in the large domain which he had to review, many aspects were outside of his competence, and he would be incapable of discussing some of them. A second aim was to be as complete as possible, but in this aim he was limited by the number of bibliographic citations (at maximum 200) which he was allowed to use. Thus he had to mention only a part of the literature published in this field. Only a few works on the following subjects will be mentioned: parasitology, clinical aspects of infectious diseases, industrial applications of microbiology, use of antibiotics in practical medicine, and practical methods of diagnosis. The author hopes he has seen most of the periodicals where articles on the subject of this review have been published; but some publications which appeared in other journals may have been overlooked. He has not been able to consult monographs, books, and treatises published in the U.S.S.R. of which one finds criticism, some of them very severe, in the periodicals. Thus these publications will not be cited here. The author apologizes for all these omissions and for the fact that this article is more a catalogue than a review.

INTRODUCTION

One of the main characteristics of the literature under review is the predominance of works undertaken for an immediate practical purpose. On the other hand, certain themes or subjects which are very popular in occidental countries have not seemed to interest scientists in the U.S.S.R. On the contrary, certain other problems which one finds only occasionally in occidental literature have been the subject of publications in the U.S.S.R.; for example, the role of the nervous system in infectious pathology and in immunology. Parts of these articles are published as answers or "contributions to the discussion" of a problem. Others are answers to questions proposed by scientific societies or editors of a journal, and there are often very different opinions and strong mutual criticism.

Since this review is the first of its kind to appear in the *Annual Review of*

¹ The survey of literature pertaining to this review was completed in December, 1955.

² The author expresses his thanks to Mrs. Nina Grabar and Miss Ludmila Grabar for their contribution in assembling the bibliography and to Mrs. Helene Miller for her help in correcting the English text of this review.

Microbiology, it was thought useful to give some short indications of the principal journals which, as far as we know, publish articles on the subject of this review. Three journals are entirely specialized: the *Journal of Microbiology*, *Epidemiology*, and *Immunobiology*, the *Microbiology*, and the *Ukrainian Journal of Microbiology*. The general journals, such as the *Proceedings* and the *Bulletin* of the Academy of Sciences and of the Academy of Medicine, or the *Journal of General Biology*, as well as the journals on related branches of Medicine, Veterinary Sciences, Biology, Agriculture etc., also contain articles on microbiology, as well as the two biochemical journals, one in Russian and one in Ukrainian.

Some general reviews are also published in these journals, but a special journal, *Progress in Actual Biology* (*Uspekhi sovremennoi biologii*), publishes general reviews. Two special journals publish abstracts of papers from world literature: one in microbiology (*Sovietskoe Meditsinskoe Referativnoe Obosrenie: Seria: Mikrobiologia, Infektsionnyie Bolezni, Epidemiologia*, three numbers per year), the other in biochemistry (*Referativnyi Zhurnal, Seria Biologicheskaya Khimia*, 24 numbers per year).

The *Journal of Microbiology, Epidemiology, and Immunology* generally publishes in the same issue a series of articles on a given problem. Some of these publications are mentioned below, but it seemed useful to stress here that in 1955 studies in epidemiology are concentrated mainly in Issue No. 1. The publications on the "natural epidemiological foci" are concentrated in Nos. 3 and 4. The problems of induced or natural variations and dissociations are grouped in No. 8. A series of publications on dysentery is to be found in Nos. 3 and 10, on rickettsiae in Nos. 6 and 7, on children's diseases in No. 9, on tularemia in No. 4, and on tetanus in Nos. 5 and 9. In addition to these principal subjects, each issue also contains articles on other questions.

Most of the publications on the microbiology of soil and of water, as well as on its applications to agriculture, appear in *Mikrobiologia*.

The delay in publication seems to be from 6 to 12 months. Therefore, most of the manuscripts of the publications under review were submitted in 1954.

SYSTEMATICS, MORPHOLOGY, VARIATIONS

(a) A new microbe, provisionally designated SB, has been isolated by hemoculture from 42 per cent of epidemic hepatitis cases (1). It could be cultivated only in symbiosis with sarcinae, by the method of Voronkova. The isolation of this microbe could be achieved in only 3.8 per cent of 102 other patients with different illnesses used as controls, and in none of 50 healthy volunteers. It seems that this germ is not considered to be the agent of hepatitis, but its isolation is a complementary diagnostic method.

A microorganism which is capable of utilizing formic salts and of reducing CO_2 to CH_4 in the presence of gaseous H_2 has been isolated from petrol-soils. It resembles *Methanobacterium formicum*, Schnellen (2). Some new strains of paracolon bacteria incapable of decomposing mannite have been described.

Thermophile microbes, which grow at 90–95°C., have been found in hot springs on Kamchatka.

The progressive transformation of the "coccoidal" terminal cells of *Bacillus subtilis* into normal bacillary forms has been studied. It is a regular mechanism and seems to correspond to a polarity in the cell, which is also of importance in the formation of the spores (3).

Polymorphism of the cells of *Mycobacterium tuberculosis*, even in the same preparation, is observed by electron microscopy. The cell membrane is thin and is destroyed when the microbe is dead. The role of intracellular granules has not been elucidated (4). The preservation of virulent strains of this microbe for long periods of time can be achieved by heavy seeding in a blood medium (5).

In a review on the nature of virus and phage particles, Ryzhkov (6) develops arguments in favor of a hypothesis that the virus particles which can be isolated are only one of the forms of the developmental cycle. It could be the more resistant phase and be compared to spores ("virospores").

Experiences with some 60 species of butterflies and two polyedric viruses (*Borrelinae*) have shown that different species can be infected by both viruses, and that the dimensions and forms of the polyedres are specific for the virus and not for the host. The formation and the structure of the proteins which surround the virus particle in these polyedres are specific for the given virus and could be considered as intermediate products in the synthesis of the virus (7, 8).

Two new strains of cucumber mosaic virus, similar but not identical with the known mosaic 2 virus, have been isolated. They form different and more regular lesions on the leaves (9).

Electron microscopy has shown that crystalline inclusions of tobacco mosaic virus exist only in destroyed chloroplasts (10), but characteristic virus particles, the number of which increases with the development of necrotic zones, can be observed even in non-necrotic zones (11).

For years the problem of the "filterable forms" of bacteria has been the subject of research in the U.S.S.R. Some years ago Boshian published astonishing results on the transformation of microbes into viruses, and proposed a general theory on these transformations. This work has been controlled by a special scientific commission, the conclusions of which have been presented to the November 1954 session of the Society of Microbiologists, Epidemiologists, and Infectionists of the U.S.S.R. in Moscow. The experiments of Boshian could not be repeated, however, and his theory has been severely criticized.

A comparative study of different methods of obtaining filterable forms has shown that ageing of cultures, treatment with distilled water at 37°C., and the action of penicillin favor the vitality of these forms, and that lysis by phages gives less positive results (12, 13). The regeneration of normal forms is easier when one uses small amounts for seeding and media rich in vitamins, proteins, or microbial protein hydrolysis products. Filterable forms of differ-

ent microbes need different media for regeneration. The use of a symbiotic microbe as a nutrient (13) is not absolutely necessary, and passage on fertilized eggs has given good results in the case of hemolytic streptococci (14). Eight out of 18 cultures of regenerated forms of this microbe have shown properties similar to those of the initial strains, but differences in the respiration and phosphorus fixation rates and proteosynthesis have been noted between the regenerated forms of hemolytic and non-hemolytic strains (15).

(b) All the publications on variation, dissociation, adaptation, and mutation will be mentioned together, because sometimes it is difficult to classify certain of these works, and also because the definitions used by different authors do not always seem to be identical.

The theory of the formation of species has been the subject of many publications, some as "contribution to discussion" others giving experimental results. The leading trend in the U.S.S.R. is that a living organism and its environment form a "unit", modification of the medium can produce a modification of the living organism, and this modification can become permanent. In other words, the possibility of hereditary transmission of acquired characters is admitted. The large number of successive generations obtained in short time with microorganisms gives a particularly tempting possibility of investigations in this domain. In a recent publication on the theory of variations in the formation of microbial species, Sosov (16) particularly stresses this point. He has calculated that microbes multiply 500,000 times more rapidly than man. In the evolution of man since one million years ago, successive transformations have taken place. Thus it seems logical that microorganisms in the same interval of time could have been modified by many transformations. He recalls that in 1809, Lamarck said that unicellular organisms, being incapable of further development over the limit of their cells, are destroyed when conditions change and regenerate again in favorable conditions. Sosov concludes that there is no continuity in the microbial series, species can disappear and do reappear when conditions favorable for their appearance are realized. For pathogenic microbes these conditions can only be realized in the host. For example, he thinks that pneumococci develop in the lungs from saprophytic microorganisms when the resistance of the host is diminished by exterior conditions (cold, humidity). The microbes which appear in definite conditions adapt themselves to those conditions and cannot exist elsewhere if they cannot adapt to the different conditions. The observations that the new strains of microbes, whose appearance can be provoked, can be generally classified in the already known species are interpreted as being due to the external conditions, the same conditions giving analogous microbes (16).

Similar opinions have been developed in another review (17); but it seems that discussion of this problem is still in progress and will be one of the main subjects in the program of the next microbiological congress in Moscow in March, 1956. A review on the appearance of strains resistant to antibiotics, written by M. Barber from London, England, has been published (18). In a

footnote the journal points out that the review appears as a "contribution to the discussion," but that certain conceptions of the author are different from the opinions of many Soviet scientists.

Cultivation of *Shigella paradysenteriae* (Flexner) on a human serum medium with killed *Escherichia coli* has permitted the isolation of microbes whose biochemical and antigenical characteristics were those of *Shigella sonnei*, while growth of *S. sonnei* on killed *E. coli* has given a strain of microbes analogous to *Alcaligenes metalcaligenes*. Analogous results were obtained, and with greater ease, by passage through the intestinal tracts of mice (19). Biochemical and immunochemical differences have been shown between fractions isolated from artificially obtained variants and the original strains, as well as the strain used as "inducer" (killed microbes). When the first obtained induced strain was further cultivated in the presence of the same inducer, the newly obtained strains more closely resembled the species used as inducer, but still had some properties of the first obtained strain. It is concluded that some genetic relationship between the induced strains must exist, that the antigenic structure is similar, but non-identical, and that deoxyribonucleic acid of the nucleoproteins plays an important role. The cells contain major constituents which are specific for large systematic groups and some other constituents which can vary and which may be specific for a particular form or strain (20).

Paper chromatography has been used to study the amino acid distribution in S and R strains of *E. coli*. Mainly quantitative differences have been observed: larger amounts of histidine in S forms, and of proline and alanine in R forms. Hydrolysates of the proteins of the R form have no cystine (21).

Atypical strains of enteric bacteria have been isolated from patients, from water, or after "regeneration" of filterable forms. They were often pigmented (yellow). The passage of these strains on different culture media or in animals, sometimes permitted return to typical strains (22, 23).

Several publications were concerned with modifications provoked by drugs. Here are some examples. Dysentery bacteria which are resistant to sulfamides are also more resistant to other actions, such as drying (24). The dynamics of the formation of nucleic acids during the growth of *E. coli* were modified when the cells were cultivated in the presence of sub-bacteriostatic doses of an antibiotic (grisemin). The decrease in concentration of nucleic acid during the phase of intense multiplication is slower in the presence of this antibiotic (25). Small amounts of streptomycin can activate the growth of *M. tuberculosis* (strains DT and Vallée) on the synthetic medium of Model. Increased yield, increased oxidation of glycerine, a decrease in content of lipides and of P derivatives, and an increase in polysaccharide content have been detected (26).

Kuragina (27) has compared the virulence and the morphological and antigenic properties of diphtheria bacilli submitted to the action of antibiotics (penicillin and gramicidin) in culture and in carriers. She has often isolated "diphtheroid" strains which had some common antigens with typi-

cal strains. In another publication (28) it is said that the passage in an animal can sometimes transform strains of *gravis* and *mitis* into pseudodiphtheria, and vice versa. The passage of a *gravis* strain in an immunized guinea pig transformed it into a pseudodiphtheria strain.

Markov *et al.* (29) have attempted to reestablish sensitivity to penicillin in strains of staphylococci resistant to this drug, by the use *in vivo* of antipenicillinase sera or by injection of penicillinase into animals. These animals have shown a higher resistance to these strains. Studies on the phagocytosis of streptomycin-resistant or sensitive strains of *M. tuberculosis* have been achieved by Lentsner (30). Differences in the pathological effects observed in mice infected with *Salmonella typhimurium*, with and without antibiotic treatment, and the role played by the host have been described (31). Freshly isolated *Brucella* sometimes present atypical properties, and do not grow in the absence of high concentrations of CO₂. The addition of an antiphage serum to the agar medium enabled growth to occur in the second passage without excess of CO₂ (32). Similar observations have been made by Uvarov (33), who has observed dissociations of *Brucella* when cultivated in classical media. The addition of serum from a convalescent individual has allowed dissociated strains to regenerate and preserves strains in a perfect state for months.

Variations induced by external conditions, such as soil and climate, have been studied by Krasil'nikov and his school (34, 35) using bacteria from the roots of *Leguminosae*. Under the action of extracts of those roots new variants, often stable, have been obtained. A review on variations in phytopathogenic bacteria which produce different pathological effects in plants has been published (36).

Variants which are artificially induced or obtained by selection, and which possess practical industrial interest, have been described. For example: variants of *Clostridium acetobutylicum*, obtained by passage on media containing salts of butyric acid, give better yields of butyl alcohol. These variants are stable (37). The selection of *Streptococcus lactis* strains which are not influenced by the seasonal variations of the composition of milk, has permitted their continuous use in the lactic acid fermentation (38).

Variations in viruses have also been studied. Differences in the virulence of vaccinia virus have been observed when cultivated on different media, but the antigenic structure was not modified. The best results were obtained when grown on the chorioallantoic membrane (39). The existence of two types (Scandinavia and Liverpool) of influenza virus A₁ has been confirmed, and the phases P-Q-R have been observed. For Gorbunova (40), these phases of Mulder, and the phase O and D of Burnet are not adaptive variations under the influence of a specific immunity factor, but adaptations to a new medium: chicken embryo, tissue culture, laboratory animals.

A mixture of three stabilized strains of plant viruses was inoculated into susceptible plants. In most cases Sukhov (41) observed that one of the strains (the thermoresistant) returned to the initial form, but in some cases

new variants were obtained, characterized by typical forms of inclusions. These variants were stable after three passages. The appearance of these new strains is explained by the intimate contact of particles of two different viruses during reproduction, thus permitting an exchange comparable to a vegetative hybridization or to a sexual reproduction in higher organisms.

Isolation of selected strains of molds and yeasts and of variants possessing practical interest have been described: examples are modification of medium for the selection of thermophilic strains of yeasts, adaptation to antiseptics to control contaminations by bacteria, etc. Imshenetskii (42) and Kasatkina (43) stressed the parallelism between the morphological appearance and the physiological or biochemical characteristics of different variants of molds.

Free sulfhydryl groups in yeasts are increased by x-ray irradiation (30–40,000 r). With larger dosage (120–150,000 r) the quantity of these groups can be doubled. This increase was maintained for 100 generations, but decreased afterwards (44).

BIOLOGY OF MICROORGANISMS

Metabolism and enzymatic activity.—The quantity of CO_2 needed for the growth of heterotrophs (*Pseudomonas fluorescens*, *Proteus vulgaris*, and *Bacillus subtilis*) is different and depends on the nature of other available carbon compounds. It is maximal when these compounds contain 3 or 6 carbon atoms. Dicarboxylic acids with 5 or 6 carbon atoms diminish the need for CO_2 , and with complex media this requirement is still lower. The CO_2 -replacing substance is neither alanine nor acetic acid, a purine nor a pyrimidine (45).

The redox potentials of culture media for *Clostridium sporogenes* and *C. putrificus* have been studied and the necessary level for multiplication and the germination of spores has been established. These microbes are capable of overcoming initial high rH, and with washed suspensions no inhibition of active growth was observed (46).

Growth of *Salmonella typhosa* and of *Shigella paradysenteriae* Flexner is strongly influenced by the aeration "in depth" of the culture medium (1 l. of air/min./l. of medium). Growth was increased up to 20 times, the number of living cells/ml. was greater, and if the inoculation was made with a few cells, the latent period was shorter than under usual conditions. The log phase was characterized by a very rapid rate of multiplication and was shortened. In the case of typhoid bacilli nearly 100 per cent of the cells were living, and in the case of dysentery bacilli about 70 per cent (47).

Several publications have described bacterial enzymes, their presence, activity in different conditions, practical use, etc.

Oparin *et al.* (48) admit that invertase may exist in the cells of certain yeasts (*Saccharomyces*) in an inactive state and that its presence can be shown after ultrasonic treatment or autolysis by toluene. Maltase, on the contrary, is in an active state and is localized in the protoplasm, but not in the cell membrane, where the invertase seems to be concentrated. During

prolonged adaptation of *Saccharomyces globosus* to sucrose the quantity of invertase increases as is also the case for maltase during the adaptation of *Saccharomyces paradoxus* to maltose. One of the forms of adaptation would be the modification of the permeability of the cell for the substrate.

The urease of *Micrococcus ureae* and of *P. vulgaris* is intracellular and may appear in the surrounding medium only after the lysis of the cells. The hydrolysis of urea must thus be an intracellular phenomenon (49).

Small amounts (0.03 per cent) of nitrites or nitrates inactivate the carboxylase of *E. coli* through destruction of its amino groupings. There is therefore no formation of ethanol or acetaldehyde and an accumulation of acetic and formic acids occurs without evolution of H_2 and CO_2 . This seems to show the importance of the $-NH_2$ groups in carboxylase and can be of practical use in the fabrication of "Dutch" cheese (less formation of gas bubbles) (50).

Some toxic substances and antibiotics (streptomycin, isonicotinic acid) inhibit the diaminoxidase of acetone dried cells of an acid-fast saprophyte *B.*, but the results have been considered by the authors as being insufficient to prove that the antibiotic activity of these drugs is due to this inhibition (51).

Increase in phosphorylase and amylase activity has been noted when *Clostridium perfringens* is cultivated on carbohydrate rich media. This phosphorylase, as distinguished from those of the muscle and of the potato, is capable of hydrolyzing maltose by phosphorolysis (52).

Using different techniques of culture, the role of the proteases in toxinogenesis by *Corynebacterium diphtheriae* has been studied. These proteases are activated by cysteine, Fe and glucose, and there is a parallelism between the appearance of the toxin and the protease activation. There is no toxin formation when *o*-oxychinolin, which inhibits the proteases, is added to the medium (53). The amino acid distribution in some fractions of the proteins of *C. diphtheriae*, PW 8, has been determined by paper chromatography (54).

A review on carotenoid pigments, from bacteria and molds, their metabolism, synthesis and known functions has been published (55). The isolation, spectrography, and photochemical action of a porphirin of *Rhodospirillum rubrum* cultivated in anaerobiosis have been described. This substance may be an intermediate in the synthesis of cytochrome and of bacterial chlorophyll (56).

It can only be mentioned here that many articles were published in *Mikrobiologia* on such subjects as: industrial alcohol and acetone-butyl alcohol fermentations; oxidative actions of *Acetobacter suboxydans*, the production of citric acid, etc.; and the role of different media, temperature, aeration, etc., in industrial installations.

The culture of vaccinia virus in developing chick embryos has shown that for the production of large quantities and for diagnostic purposes different techniques may be used. Inoculation of the chorio-allantoic membrane is preferable for the detection of viruses, but the infection of the allantoic

membrane is simpler and resulted in fewer killed embryos (57). The possibility of cultivation of spring-summer encephalitis and Japanese encephalitis viruses has been confirmed, and a technique giving optimum conditions for each virus, and the relative concentrations of virus in different parts of the embryo have been described (58).

The infection of tobacco and tomato leaves with a virus stimulates dehydrases and peroxidases and diminishes the activity of catalase (59). Glutamic acid and cysteine inhibit phage multiplication, and also the phosphorus metabolism of *S. lactis*, either in resting or growing cells (60).

The action of bactericidal and antibiotic substances.—Use of radioactive-tagged molecules has indicated that sulfamides are concentrated within the bacterial cell. The intracellular content is higher when the external concentration is increased. Para-amino benzoic acid does not prevent this accumulation (61).

Antibiotic activity against the cholera vibrio can be measured in the developing egg by the survival of the embryo and by pathological modifications of its organs and membranes (62). A comparative study of antibiotic activities among different groups of the genus *Penicillium* has shown that the more complicated the morphological structure of a mold, the more chances there are to find an antagonistic effect against bacteria. The largest proportion of strains active against the test microbes (*Micrococcus pyogenes* var. *aureus*, *B. subtilis*, and *E. coli*) was found in the *Assymetrica*, and the smallest among strains of *Monoverticillata stricta* and *Biverticillata symmetrica* (63). The morphological modifications produced in *Bacillus mesentericus* by six different antibiotic substances extracted from *Actinomyces* are not identical (64). Many other studies on the action of different antibiotics have been published, mainly in the *Journal of Microbiology, Epidemiology, and Immunobiology*. Here are some examples: action of albomycine (with or without penicillin) on gram positive and gram negative microbes; action of syntomycine on *P. vulgaris*, of different antibiotics on the cholera vibrio, of penicillin on diphtheria bacilli, of biomycine in whooping cough; on the combined action of ecmolin and penicillin on penicillin-resistant strains of staphylococci; on the useful effect of leucine on the action of levomycetine on enteric microbes; on the use of aspergiline, syntomycine and furacilline for dysentery, on the use of antibiotics in rickettsial diseases. The recently purified furacillin is a nitrofurane. It acts on acid-fast and on gram positive and negative microbes. Its activity range seems analogous to that of streptomycin (65). To this simple enumeration, some special studies may be added: a favorable effect has been observed in dysentery patients following the introduction into the digestive tract of very large amounts of strain M 17 of *E. coli* (66). Preparations and therapeutic use of phages adapted to freshly isolated and local strains of dysentery bacilli have been described (67), as well as the use of antibiotics in combination with phage therapy against dysentery in children (68).

Microbiology of soil and water.—The large number of publications on this

problem has incited the author to review them in this special section, although many of them could be mentioned elsewhere.

A series of reviews, published in *Mikrobiologia* as a "contribution to discussion", concerned the problems of the ecology of soil microbes and their role in the fertility of the fields. Only titles of these publications can be mentioned here: Geller: The biological factors of soil fertility; Seliber: The problems of soil bacteriology in connection with the actual state in other domains of ecological microbiology; Mishustin: Microbial associations and the means for their study; Rubenchick: Some discussed problems on the role of soil microorganisms in plant nutrition; Aristovskaia: About the discussion of the problems of soil microbiology; Voznekovskaia: Fertilizers and the soil microflora; Santsevich: Periodicity and the influence of seasons on the development of microorganisms in the soil.

A study of the ecology of *Bacillus megaterium* in the soil has shown its wide distribution, its particular abundance in cultivated fields, and its existence in various morphological forms (69). Experimental results suggest that soil bacteria not only furnish nutritive substances to plants, but may also increase the absorption of these substances by the roots (70). The presence of different *Actinomyces* in different soils, their antagonistic role and the persistence of the antibiotic substances produced by them in natural conditions have been studied (71, 72, 73).

The microflora of the rhizosphere of the oak (74, 75), of young pines (76), and of bushes (77) are particular associations with predominant species present in different seasons. Some species of bacteria and molds are common in most soils. Some other species are specific; such are, *Bacillus aerosporus*, *Bacillus danicus*, *Bacillus asterosporus*, and *Bacillus teres* in woods, and *Bacillus petasites* and *Bacillus ruminatus* in the steppes (75).

Several publications relate studies on the microflora of water (rivers, lakes, seas) and of slimes. In the region of the North Pole, for example, numerous cocci, bacteria, and yeasts have been found in the water and in the deposits on the bottom of the sea, showing an intense activity even in these conditions (78). The extent of synthesis in slimes and in bogs has been measured by the use of radioactive carbon. It is of the order of 4-6 mg./kg. of moist grey slime and of 0.1-1.2 mg. for the slime in bogs (79).

A number of papers on *Azotobacter* appeared in *Mikrobiologia*, and deal with its presence or absence in different soils, on the action of salts or acids in the soil, on atypical variants, etc. Thus, for example, Fedorov and Ernand found that atypical forms which fix only small amounts of nitrogen can be activated by yeast autolysate or by molybdenum or boron. These authors suggest that the atypical strains may correspond to a degradation stage of *Azotobacter chroococcum*, induced by conditions of life in acid soils poor in carbon compounds (80). A colorless variant of *Azotobacter* isolated in Siberia can fix nitrogen even at pH 5.5; that is, in conditions where the normal strain cannot grow (81).

Imshenetskii considers *Nitromonas* as the sole agent responsible for the

first phase of nitrification, and *Nitrosocystis*, *Nitrosospora*, *Nitrosogloea*, etc. are simply accompanying species. He thinks that mixed cultures of *Nitromonas* with microbes of the genus *Sorangium* can better preserve their activity of nitrification (82). The control of purity of *Nitrosomonas* is rendered difficult because some of the accompanying species are similar in morphological appearance (83).

The results obtained in recent years confirm previous statements that ammonia is the first step in nitrogen fixation by *Azotobacter* (84). Pure cultures of bacteria from the nodules of *Leguminosae* have fixed nitrogen when cultivated in hay-broth with glucose or inverted sugar (85). Further studies on the oxidation of ammonia and the formation of nitrites by filtered autolysates (cell-free) have shown that this transformation is limited and that the initial increase of nitrite is followed by a decrease, the total nitrogen staying constant (86).

INFECTIOUS DISEASES

As previously mentioned, a series of reviews and "contributions to the discussion" have been published in the *Journal of Microbiology, Epidemiology, and Immunobiology* on general problems of epidemiology or infections. The principal articles are: in Issue No. 1, Diadichev: the definition of the term "infection"; Bunin: the rules for the establishment of a classification of infectious diseases; Nosov: the classification of infectious diseases; Shreiber: on the methods of epidemiologic investigations; and three articles on the theory of epidemiology by Guslits, by Rozhdestvenskii and Agafonov, and by Elkin. In Issue No. 7, Znamenskii and Beliakov discuss the same problem. Four articles on the "natural foci" of infectious diseases have appeared in Issue No. 4. The first of these reviews is by E. N. Pavlovski who studied these "natural foci" for many years, and who organized many expeditions in different parts of the U.S.S.R. for their study and eradication. He concludes that the factors involved in the formation of such foci are climate, soil, insects and other parasites which transport the pathogenic agent, and wild animals on which such parasites live, and stresses the necessity to combat several of these factors to control epidemics.

Epidemiological studies on tularemia have shown that the "foci" may be aqueous (87), that the infection may be transmitted by ticks (*Dermacentor*, *Hyalomma*) (88), and that for controlling tularemia it is necessary to destroy small rodents and to vaccinate and revaccinate the inhabitants of the area. The existence of "natural foci" in brucellosis, and the ways of its transmission could not be established (89).

The role of endogenous factors in the pathogenesis of bacterial infections has been reviewed by Planel'es (90) who has studied this problem with his collaborators. He insists on the importance of the action of histamine liberated in infected organs (See also 91). The use of antihistamine drugs, for example, could, to a certain extent, protect animals locally infected with pneumococci. This is explained as being due to a decrease of the exudate

thus making conditions less favorable for the multiplication of the microbe. Other cases of endogenous reactions which may defavorize the host are also considered: modifications of internal secretions, activation or inhibition of certain enzymes, etc.

A large number of publications has been devoted to dysentery and related infections, whose agents are often considered as atypical or variants. Special attention is paid to "carriers" and the microbes dispersed by them. The Newcastle bacillus has been classified as a sub-species of *Shigella dysenteriae* Flexner; the clinical and epidemiological aspects of the disease produced in man by this microbe have been described, and 96 strains of *Shigella*, type Boyd, have been studied (92). A gas-forming variant, but having the same antigenic structure, has been regularly observed (92a).

Strains of *S. typhosa* isolated at different periods of the infection in man contained variable proportions of Vi-antigen, and no correlation could be established between these proportions and the clinical form of the disease, its severity, or its different stages (93).

Studies of the microbial flora in hematogenic osteomyelitis have shown that strains isolated from the blood are often variants of streptococci or staphylococci present in the infected locus, and it is suggested that variation has been induced through the action of antibodies and drugs. These strains are sometimes penicillin-resistant, but not gramicidin S-resistant (94).

The question of "normal carriers" is also invoked in the studies on epidemiology of diphtheria and scarlatina (95). Different types of scarlatinous hemolytic streptococci and their capacity to form hyaluronidase, fibrinolysin and streptolysin have been studied (96).

Serratia marcescens (*B. prodigiosum*) is considered to be a saprophyte but it may induce fatal infections in feeble silkworms. The penetration of the microbe into the intestine or through wounds of the cuticula in those cases may give rise to a septicemia (97).

Epidemiological studies on Q-fever have shown its higher frequency in land areas than in towns (98), that ticks can be the infecting agents (99), and that cattle may transmit rickettsiae to man, the germ being present in milk and in the dust of stables (98,100). The guinea pig is a convenient animal for experimental work and the isolation of strains. Histological studies have been published (101).

Among publications on virus induced diseases, a general review on epidemiology and prophylaxis of infectious hepatitis (Botkin's disease) needs to be mentioned (102). An immunochemical method has been elaborated for the diagnosis of this disease and is based on the observation of a precipitin reaction given either by the patient's serum with an extract of livers from fatal cases, or by a chicken serum anti-hepatitis liver with the serum of hepatitis diseased persons (this serum would thus contain some antigenic constituents from the destruction of the liver.) The reaction is not considered to be strictly specific for this disease, and other observations must help

diagnosis (103). In the early stages of this disease a 4 to 8-fold increase in aldolase activity of the serum has been observed and has led to the use of the determination of aldolase as a rapid diagnostic test, because such large increases have not been observed in other diseases (104).

A virus from epidemic gastroenteritis has been isolated. Its pathogenic effects in man are not severe. Newborn, suckling mice have been used for passages of the virus (105).

An epidemic of influenza, type C, has been observed in children. Some antigenic relationship with the Newcastle disease virus has been found. Normal rat serum contains an inhibitor for the agglutination of chicken erythrocytes by this virus. A preliminary diagnostic use of this observation is envisaged (106).

The resistance of albino mice to the fixed virus of rabies can be diminished if the animals are submitted to various conditions: fatigue, cold, alcohol (107).

The present author has thought it logical to include a certain number of studies on cancer in this section, because it seems that the authors of these studies follow the idea of a viral cause of this illness. For many years Zil'ber and his collaborators have published interesting results of their immunological research on tumors. Some twenty papers appeared since 1949, including a review on their work (108). Desensitization for anaphalactic shock is used as a standard method for the control of the specificity of different fractions extracted from tumors. Fractions containing different nucleoproteins have been isolated from tumors at different periods of their development. In 1955, Zil'ber *et al.* (109) studied a rat's sarcoma M₁, nontransmissible by filtrates. They found again that a specific substance is present in nucleoprotein rich fractions, such as mitochondria, microsomes, and a fraction called by them "glubulin I". As a contribution to the theory of viral origin of cancer, Nicolau (110) reviewed the known cases of cellular proliferations induced by known viruses.

Interesting observations have been made on tumors by the use of a technique common in virus research: culture on the developing egg. Shubladze (111) has used this technique with pieces of human and animal malignant tumors and leukemic blood, and as controls, employed pieces of nonmalignant tumors, of normal human tissues, and of mice brains infected by herpes and encephalitis viruses. From numerous experiments it can be said, in short, that often (15 to 50 per cent) these explants may enlarge 200-fold in seven to nine days of incubation. Human tumors could support up to 30 passages from egg to egg, but in the case of Ehrlich's mouse carcinoma, the pathogenicity for mice vanished after three to five passages, although antigenicity (complement fixation) still persisted. Filtered tumor or tissue extracts did not produce modifications on the chorio-allantoic membrane. Microscopical examinations showed different modifications of the egg membrane when normal or tumor tissues were used. Liver from fatal cases of hepatitis could not be cultivated. Analogous results have been obtained

(112) by applying the same technique to the culture of Ehrlich's adenocarcinoma, Crocker's sarcoma, an acridic sarcoma, and the mammalian cancer of mice. The specificity of the transplants decreased after five to ten passages on eggs. Some histological modifications of the chorio-allantoic membrane are considered to be specific.

The possibility that the "milk factor" of mice can persist in a dried state in natural conditions is stressed by Medvedeva (113) who has observed that 59 per cent of dried preparations gave positive results after one year and 20 per cent after two years of preservation.

IMMUNOLOGY

Phagocytosis.—Phagocytosis or the phagocytic index has been the subject of several publications: description of techniques (114), applications to diagnostic purposes, or in experimental infections, such as dysentery, tuberculosis, and diphtheria.

If one uses typhoid bacilli on which vaccinia virus has been adsorbed, the leucocytes of a rabbit immunized against this virus have a higher phagocytic index for such a suspension than for a simple suspension of this microbe, attributable to the opsonization by the serum (115). Microbial toxins may act on the phagocytes. Thus *Clostridium oedematiens* toxins inhibit phagocytosis. Neutralization of this inhibition is obtained with homologous immune serum, but only partially by an anti-*Vibrio septicum* serum. The action of leucocidins is only partially suppressed even by the homologous serum (116). Quantities of botulinus toxins A and B, even smaller than those which can be detected by the usual methods, modify the phagocytic index. This technique is rapid and can be used for the detection and eventually for the determination of the type of toxin; for example, in the control of canned products (117).

Generalities. Antibodies.—A posthumous article of Gamaleia (who died in 1949, at the age of nearly a century, and who was the last surviving direct collaborator of L. Pasteur) has been published. He insists on a "third factor" in immunity (the others being phagocytosis and antibodies), the tissue constituents, which he named "tissulins" and which would have mainly a lytic action on microbes, comparable to the action of lysozymes or phages, or to the action of an enzyme, lecithinase. The case of tubercule bacilli is cited (118).

A "resistant" to coccidiosis (*Eimeria perforans*, *Eimeria stidae*, *Eimeria magna*, and *Eimeria media*) second and third generation of rabbits has been obtained by coupling experimentally selected resistant males and current females (119).

The important role played by lymphoid cells in antibody formation has been confirmed, using *Salmonella typhimurium* antigens. The plasmacytes are considered as a morphological expression of antibody synthesis and two successive steps are distinguished: the transformation of corpuscular anti-

gens by the cells of the endothelio-macrophagic system into soluble substances and the action of these on the lymphoid cells (120).

Ultraviolet irradiation (different wave lengths have been used) during immunization seems to increase antibody (agglutinins) formation. A simultaneous narcosis would permit the obtaining of higher titres (121).

The method of antigen injection in the production of hemolysin by the rabbit has been studied, and the best secondary response after the injection was obtained in the following decreasing order: into the carotide, intravenous, subcutaneous (122).

The bactericidal activity of human and animal skin is less pronounced against bacteria normally present on the skin than to "foreign" microorganisms. External conditions, such as cold or hot weather may change this activity. Hyperergic reactions of the skin following the injection of substances such as milk, peptone, or turpentine have no effect. Local anesthesia decreases and strychnine increases this bactericidal activity (123).

Therapeutic and prophylactic utilization of hyperimmune anti-spring and summer encephalitis serum has shown its practical value. Different methods of purification have been tried. Fractionation by dialysis has given inconstant results; enzymatic treatment occasioned losses in activity; while alcohol fractionation has allowed a 23-fold increase in titre (124). The same method has been recommended for obtaining γ -globulins from anti-measles sera (125).

Antigens. Vaccinations.—Several publications treated the questions of the use of diphtheria and tetanus purified and adsorbed toxoids for immunization of children, the observed reactions, protection, etc. Hypovitaminosis C is said to inhibit diphtheria immunization and to diminish acquired immunity (126). The logarithm of tetanus toxoid units adsorbed per mg. of aluminum hydroxide is directly proportional to the log of toxoid units per mg. of total N, hence it is possible to diminish the doses of hydroxide when purer preparations are used (127). Shorter culture times (36 hours instead of 10 days) are sufficient to obtain analogous titres of diphtheria toxin, if agitation is used. Isoelectric precipitation is proposed as a simple method to separate toxin from bacterial proteins, but it seems that these purified products are less antigenic, although the small amount of impurities still left has no inhibitory effects on antitoxin formation (128). Injection of large doses of antigenic substances (horse serum) into young dogs inhibits the formation of natural diphtheria antitoxins, while in rabbits the same injections made during active immunization produced an initial stimulation followed by a decrease in antitoxin formation (129). In associated vaccinations (diphtheria-tetanus or the same with dysentery), antibody production was analogous to the formation of antitoxins in response to each toxoid injected separately (130). The injection of a pentavalent vaccine has provoked a decrease in diphtheria antitoxin level in those school children who had been immunized by toxoid four years before (131).

Whooping cough prophylaxis, its treatment, particularly with antibiotics, the isolation and properties of toxic substances from *Hemophilus pertussis*, etc. have been described in many articles. The use of aeration to obtain abundant cultures, the choice between recently or non-recently isolated strains, and the importance of the volume of the inoculum have been studied. Cultivation in cellophane bags with strong aeration gave a superior yield (132). The immunogenic value of pertussis vaccine has been studied using sera of rabbits injected with these vaccines. These sera are introduced intranasally to mice which are afterwards infected by the same route. The best protection for a large infective dose has been obtained with sera of rabbits immunized with living microbes. This was preferable to those immunized with formalized vaccine or those who received heat killed bacteria. The absorption of potent sera by these killed bacteria diminished their protective value. It could be reestablished by adding a small amount of the original serum, although this amount per se was insufficient for protection. The addition of thermolabile antigens to the vaccine is therefore recommended (133).

Albino rats can be used in experiments on *H. pertussis*. They are particularly sensitive to the endotoxin, but the injections must be made intraperitoneally (134). Several groups of investigators have prepared and studied the actions of the different toxic substances of *H. pertussis*. The necrotic action on guinea pig skin has been used as a test for the choice of strains. The corresponding toxin is not completely inactivated by the action of formalin. It is labile and precipitates at pH 4-4.5. It is antigenic even after the detoxication by 0.4 per cent formalin, and its addition to heat killed vaccine protects the animals (intraperitoneal injections to rats or nasal infection of mice), although each product used alone is less active (135). The purified labile (destroyed at 56°C.) toxin had no hemagglutinating activity. Its intradermal injection produced locally a spasm of the vessels, followed by a necrosis, and this action was absent in animals previously immunized with this toxin (136). The hemagglutinin was also labile, but could be preserved in 50 per cent glycerol. It can be extracted by 1 M NaCl and separated by adsorption on erythrocytes or stromata (particularly with human O group). The injection of such an adsorbate into a rabbit permits the development of a potent anti-hemagglutinin serum. A method for the elimination from serum and egg-white of nonspecific inhibitors of hemagglutination has been described (137).

The problems concerning scarlatina are treated in several publications. Negative results have been obtained in the search for "normal carriers" to explain contaminations (138). Prophylactic action of γ -globulin for children in contact with scarlatina cases is confirmed. The Dick reaction was used as a control (139). Experimental vaccination of rabbits and guinea pigs with an associated preparation, antidipteria-antiscarlatina, has shown that both constituents act as if they were used alone (140). A method of preparation of a streptococcal-scarlatinal toxoid has been developed. Using cultures

in cellophane bags, a toxin of 3×10^6 cutaneous doses/ml. has been obtained (141). The detoxication by 0.1 to 0.5 per cent of formalin of preparations purified by alcohol fractionation is rapid at 39°C ., but it continues even when preserved in the cold. Loss of antigenicity is occasioned by the formalin treatment, but this loss is slower than the loss of toxicity. Antigenicity has been determined on rabbits by the combination rate with antitoxin (142). The allergic properties of the toxin have also been studied (143). A precipitin test using the urine of the patients and an immune anti-streptococcus, type specific, serum has been envisaged for diagnosis purposes (144).

A special journal, *Problemy Tuberkuloza*, contains publications on tuberculosis, but most of the published articles concern clinical, anatomical, histological, etc. aspects of the problem. A general review by Togunova (145) on tuberculosis vaccination appeared in that journal. Results obtained by parenteral, cutaneous, and intradermal vaccination of guinea pigs are compared. It is confirmed that the BCG strain is immunogenically a complete vaccine, that its use in dermal or intradermal vaccination is harmless and sufficiently effective. The dosage used now in the U.S.S.R. for intradermal vaccination of children is somewhat smaller than that used in other countries (0.1, 0.05 and 0.01 mg. instead of 0.2–0.5 mg.), in order to avoid abscesses (145).

The study of the dissemination of BCG introduced in two forms (liquid and dried vaccines) by scarification in the skin of guinea pigs and in different doses has shown that, in the mean, the bacteria are at their maximum concentration in local lymph nodes two to three weeks after their introduction, and in two to three months the concentration is lower. Bacilli have been found even in the bone marrow and the kidney. This proves that the dissemination is very profound even after a cutaneous introduction. Nine months later very few microbes persisted in the organs. Increased numbers of bacteria seemed to present no advantages and a standard concentration for liquid vaccine of 20 mg./ml. is recommended. The use of the same dosages with dried vaccines has given analogous results, but a certain delay in the appearance of microbes in the organs and in lymphatic nodes has been observed. Living bacilli have been found in organs eight months after vaccination (146). Experiments on albino mice vaccinated with BCG have shown that a single introduction (dermal, subcutaneous, or nasal) of the vaccine confers only a feeble resistance against an infection with a virulent strain, but three intraperitoneal injections at eight-day intervals produce good resistance. This last schedule is proposed as a test for the control for vaccine preparations (147). No essential differences have been noted when BCG was given to newborn children by intradermal or oral routes (148).

Several series of publications concern prophylaxis of enteric diseases, mainly dysentery and typhoid fever. A review on dysentery control has been published by Zhdanov (149). Much of the experimental work was

directed toward the choice of strains usable for vaccination purposes, the relations between the protective value of a given strain, its virulence and antigenicity, the variations observed in the freshly isolated or preserved strains, the preservation of strains in different conditions, and the differences in the protective value depending on the time since their isolation. Highly virulent and freshly isolated strains are often more immunogenic, but even among them only part (11 out of 53 tried) has given satisfactory results (150). A medium with gelatin permitted the preservation of strains for three years without loss of virulence (151).

Dysentery and typhoid bacilli treated with cobalt sulfate (0.01 to 0.025 per cent) are viable (particularly dysentery bacilli) but their virulence and immunogenic properties diminish, the latter still being two to three times superior to those of the formalized vaccine (152). The following method has been proposed for the measurement of the protective value of an anti-dysentery vaccine: mice are vaccinated with the preparation, then infected and the rate of disappearance of living germs from the organism is measured. In correctly immunized animals this occurs in seven to ten days (153). Intraperitoneal injection of bacteria suspended in 0.4 per cent agar for immunization control is recommended. The multiplication of the microbes is favored and the production of antibodies is not inhibited (154).

Experiments have been performed in order to compare the actual vaccines prepared from heated microbes with preparations of "complete antigens" in solution or adsorbed on alum or on calcium phosphate. In one study *S. typhimurium* and mice have been used, and the complete antigens have been extracted from the cells or from the culture medium by digestion, then purified by precipitation and dialysis. These preparations were relatively rich in nitrogen (6.5—10 per cent) and their content of reducing substances varied from 5 to 30 per cent depending on the origin and extraction technique. Equivalent amounts of these preparations or of microbial cells have been used for immunization. Serum of rabbits immunized with the complete antigen was more effective in the protection of mice against the toxic effect, but none of the serums tested had a protective effect against infection; they only prolonged the survival of mice for five days. Active immunization of mice by these preparations, on the contrary, protected them against lethal infection, particularly when infection was delayed (e.g., 40 days after immunization the survival rate was 92 to 100 per cent). The state of immunity was prolonged when calcium phosphate adsorbed preparations were used. It has been concluded that this preparation is not inferior to the heated bacterial cell vaccine (155).

In another publication on the same problem, *Shigella paradysenteriae* vaccines prepared by heating microbial suspensions were compared to "complete antigen" preparations (Boivin's method and Raistrick and Topley's method). The serum of rabbits immunized with these preparations was titrated for precipitins and agglutinins and its protective value for mice (1 MLD) was evaluated. It has been concluded that adsorbed antigens

prepared by acid extraction when used in five-fold amounts (as compared to microbial suspensions) give satisfactory results (156).

As a conclusion, based on experiments with animals, Korobkova (157) recommends anti-plague vaccination with living vaccine, that the second injection should be given a long time after the first, and that it is preferable to use cutaneous and not subcutaneous vaccination, because it is more effective, easily performed, and gives less undesirable reactions. For the immunity control, cutaneous allergic reactions are useful. In the guinea pig, parallelism between the appearance of such reactions and immunity has been demonstrated, the immunity being controlled by the resistance of the animal to infection with a virulent strain. Few cutaneous tests performed on immunized man have given positive results.

The questions of brucellosis and tularemia have been the subject of several publications. A dried living anti-brucella vaccine is in use. The period of highest prevention corresponds to two to nine months after vaccination, but even if it is performed soon before a possible contamination it diminishes the percentage of infections.

Some reactions are observed after the immunization. They can be strong in persons who have been in contact with diseased animals. Of the vaccinated persons, 0.36 per cent contracted brucellosis and these persons were in very intimate contact with highly infected animals (see per example 158).

A mixed vaccine, anti-brucella and anti-tularemia, has been tested in guinea pigs by cutaneous and subcutaneous routes and controlled by agglutinin titrations, by allergic cutaneous tests, and by protection tests against injections of virulent microbes. The cutaneous vaccination, although it produced lower agglutinin titres and lighter skin reactions, protected the animals against infections by virulent strains of *Pasteurella tularensis* and *Brucella melitensis* (159).

An allergenic extract (tularin) have been obtained from virulent and avirulent *P. tularensis* strains (160). Chemical examination of allergic fractions of *Brucella* (obtained by mild heating of cells in dilute acetic acid) has demonstrated that they are complexes of polysaccharide and protein in which only the protein moiety is allergenic. Chromatographic analysis of amino acids distribution showed quantitative and qualitative differences between these allergens and the main cytoplasmic proteins of the bacterial cell (161).

Dried anti-rickettsial vaccines have been prepared; they were still active after 2½ years of storage (162). Guinea pigs were vaccinated with a killed vaccine of Q-fever rickettsiae; three injections with eight-day intervals between gave good results. Vaccination of man with the same vaccine confirmed earlier serological observations of antibody formation and their persistence as a result of these vaccinations (163). The allergic cutaneous sensitivity appears in Q-fever cases on the ninth day and can persist one to three years after healing (164).

Histological examination of the local reaction produced by the subcu-

taneous injection of anti-typhus vaccines (dried and liquid preparations) showed that the "depot" formation is particularly strong when "dried" preparations are used. Resorption is produced by granulocytes, macrophages, and giant-cells (165). More than 80 per cent of the persons who had typhus 25 to 40 years ago still possessed opsonins, complement fixing and toxin-neutralizing antibodies in their serum and gave a positive skin test with rickettsial lysates. The most sensitive reaction was the opsonization-phagocytosis test (166).

From a study on the efficacy of two different anti-influenza vaccines it has been concluded that a mixed A, A₁ and B vaccine prepared from viruses grown on human embryonic tissue culture gave better results than a vaccine prepared without passage on tissue culture. Vaccinations of large numbers of humans by the nasal route has been performed, but in the absence of a strong epidemic the evaluation of the results was difficult. During a small epidemic of type A₁ during 1953-54 the activity coefficient of this vaccination was 2.3 to 3.2 (167, 168).

Many articles on serological and immunological methods of diagnosis have been published, particularly in enteric diseases, brucellosis, and tularemia. Agglutination, precipitation with extracted haptens, cutaneous reactions, typing with phages, and other classical methods have been employed. Some of the special methods have been described above. Two others many be mentioned here. A rapid method of rabies diagnosis using phase contrast microscopy of animal brain sections (either stained by Romanovski-Giemsa method, or without staining) to observe Negri bodies have been described (169). The second concerns the nonspecific inhibition of hemagglutination in the diagnosis of influenza. To avoid it, use of a rabbit anti-human saliva serum is suggested, which has practically no effect on the specific reaction and suppresses the nonspecific inhibition (170).

THE ROLE OF THE NERVOUS SYSTEM IN THE PATHOGENESIS OF INFECTIOUS DISEASES AND IN IMMUNOLOGY

It is natural that in the country of I. P. Pavlov, a large role is assigned to research on the nervous system. The Academies of the U.S.S.R. have insisted on this problem and favored the development of research on the nervous system in general and particularly on its role in infectious pathology and in immunology.

The actual state of the problem, the questions to be discussed, and the general directions for research have been reviewed at the beginning of 1955 by Vygodchikov (171). The author of the present review thinks that the main lines of this article can be summarized as follows: commonly the interactions between the microorganism and the macroorganism (the host) are considered to be based on chemical reactions, without taking into account the role of the macroorganism, which represents a "unity," or a total system *in se*, in pathological or immunological events. The organism reacts with all his defense mechanisms, and these mechanisms are not autonomous;

their activities are under the control of the nervous system, either directly, or in a humoral way. The leading tasks of the nervous system are: to regulate the normal physiological functions, to prevent infections, to fight against an existing infection, and to reestablish or to compensate for the disorganized functions. Two ways are seen for the further development of the problem: discussions of the known facts and accumulation of new experimental results.

Some of the publications on this subject correspond to the first of these ways; they are "contributions to the discussion" and are critical reviews of the theoretical aspects of the problem and arguments taken from previous work are used. The following is an enumeration of this type of article: Ado: Physiological research in immunology (172). Pletsitiy: Role of supplementary excitations in the development of infection (173). Ostryi: Once more on the reflex-principle in infectious pathology (174). Monaenkov: Modifications of the superior nervous activity during immunization (175). Peshkovskii: Role of the neuro-reflexive mechanisms in infection and immunity (176). Alymov and Kucherenko: The nervous receptivity and its role in immunogenesis (177). Arshavskii: The reflex-principle in infectious pathology in the light of ontogenesis physiology (178). Ado: Against the vulgarization of the "nervism" in infectious pathology (179). It would be impossible to summarize these articles and the different opinions of their authors. It may only be mentioned that all of them seem to agree on certain aspects of the problem, such as the direct effects of microorganisms or of their toxins on the nervous system. But other questions, such as the role of reflexes, are strongly discussed. Most, if not all, of these authors admit also that much more supplementary research must be done before the theoretical aspect of this problem will be assessed.

Many other publications contain new experimental results. Experiments performed for the purpose of showing the formation of antibodies under the influence of conditioned reflexes have again been repeated, but negative results have been obtained. Using as a conditioned exciting agent a heterologous antigen, no increase in specific agglutinins was observed (180). More complete conditions and the use of two different antigens and previously immunized rabbits (which had been rested for 30 days before the intervention of the conditioned excitor) also gave negative results: the antitoxic titre was not modified (181). But, if a conditioned reflex to a strong sonic excitation was established in rats, a small dose of dysentery antigen provoked a higher mortality and more severe lesions in those animals than in controls (Monaenkov, cited in 173). Karpov *et al.* (182) described the following experiments: a series of rabbits was injected with magnesium sulfate under strictly standardized conditions and during the injection a bell was rung. The other series received the same 25 injections but under conditions made markedly variable. A 26th injection was given to each of the two groups under the same conditions but with tetanus toxin (50 mice LD). The control rabbits and those of the second series had distinct local

paralysis, but not those of the first series. It was concluded that cortical impulses can inhibit the action of absolute excitants (toxin); that is, diminish the sensitivity of an animal to a pathogenic agent.

Other studies on the relations between the tetanus toxin or toxoid, their immunologic action, and the nervous system have been published. It has been observed that local anesthesia diminishes the production of antitoxin in rabbits receiving intramuscular injection of toxoid, as compared to animals treated with saline instead of the drug (183). A higher production of antibodies has been obtained in rabbits when the muscular receptors have been excited (184). The injection of 1 MLD of tetanus toxin into guinea pigs does not kill them if the injection is made in the superior part of the ear, and if it is made in the muscle of the ear base the pathological manifestations are modified, as compared to controls, and death is delayed (185). Summation of partial excitations has been admitted as a result of experiments where variations in amounts of toxin, intervals between injections, and their total number have been compared (186, 187).

Numerous experiments have been performed with other antigens and medicamentous inhibitors or excitors of the nervous system. Medicamental sleep (urethane, barbital sodium) does not protect mice from streptococcal infection (188) and inhibits the formation of anti *H. pertussis* antibodies (189); but excitation with caffeine has protected the animals and increased antibody production. On the contrary, with guinea pigs which are resistant to streptococci, the inverse effects have been noted (188, 189). If during an anti-*Brucella* vaccination mice are treated with caffeine, the antibody titre rose higher initially than in controls, but the difference disappeared after a certain time. This has been explained by an over excitation produced by prolonged administration of the drug (190). Analogous observations in other cases have been described: extension of the incubation period for vaccinia from 36 to 96 hours when drugs inhibiting the nervous system have been administered (191), delay in antibody formation (see also 123, but contra 121), lowering of the titre of complement, modifications in the leucocyte formula in peritoneal exudates, when the animals have received barbital sodium (192). Allergic reactions could be observed sooner in animals vaccinated with living *Brucella* when treated with caffeine and later when treated with bromides (193).

The action of drugs in the Shwartzman phenomenon has been investigated and it has been found that medicamentous sleep applied during the preparation phase does not modify the reaction, but protection against the final phase could be achieved by the simultaneous use of two drugs (urethane and hexenal) which act on different parts of the nervous system (194).

Anaphylactic shock can be observed if horse serum is placed on the medulla of a previously sensitized rabbit. This shock could be generally avoided by applying direct current, adrenalin, or sympathomimetin (195).

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ANTIMICROBIAL CHEMOTHERAPY^{1,2}

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Antibiotics have been employed in clinical medicine for almost a decade and a half—yet the field of antimicrobial therapy is not in a stable state. The literature dealing with chemotherapeutic agents and their use is growing at an unprecedented rate. Thus, a drug like erythromycin (of some usefulness but not of revolutionary importance), which was announced in 1952 (1), could boast over 420 titles in "its" bibliography by 1955! To cope with this flood of printed matter there have appeared many reviews, and reviews of reviews. The present summary fits into the latter category.

The extensive and superbly analytical review of antibiotics by Eagle & Saz in the *Annual Review of Microbiology*, 1955 (2), so thoroughly covered all fundamental aspects of the field that little new material can be added at this time. During 1955 there have been no striking advances in the understanding of antibiotic action, and no new drugs of established importance have been discovered. Among the dozens of antibiotics announced during the past year, a few have had striking names; but their worth remains to be proven. For this reason, it is proposed to discuss briefly in the present chapter some selected, practical aspects of chemotherapy encountered daily by the physician who uses antibiotics.

On the whole, the position of antimicrobial agents in medical therapy is highly satisfactory. The majority of bacterial infections can be cured simply, effectively, and cheaply. The mortality and morbidity from bacterial diseases has fallen so low that they are no longer among the important unsolved problems of medicine. These accomplishments are widely known and appreciated. Rather than dwell on them here, it is proposed to examine a few of the remaining problems of antimicrobial therapy. This is done in the hope that the nonmedical reader may gain some understanding of the doctor's dilemma in the daily use of antibiotics. In particular, the author wishes to call attention to the abuse of antibiotics, its causes and results; to situations where antibiotics fail to cure infection; to the role of the host in antimicrobial therapy. Finally, new drugs will be considered briefly, and a few problems of chemotherapy in staphylococcus infections and tuberculosis will be discussed.

¹ The survey of literature pertaining to this review was completed in January, 1956.

² The work in the author's laboratory, mentioned in this review, was supported by grants from the National Institutes of Health (E 214); Burroughs, Wellcome and Co.; Charles Pfizer and Co.; The Committee on Pharmacy and Chemistry of the American Medical Association; and the Committee on Research of the University of California School of Medicine.

During 1954 the production of antibiotics for medicinal use in the United States reached about 1,840,000 pounds, or, 920 tons (3), with a sales value of 240 million dollars. Assuming a total population near 160 million, there were produced approximately 5.2 gm. of antibiotics for each man, woman, and child in the United States. Exports accounted for perhaps $\frac{1}{4}$ of the production. Therefore, the consumption in the United States was about 4 gm. of antibiotic per individual. This represents a full dose of tetracycline or chloramphenicol for two to four days, or an average penicillin dose for 11 days. There must have been some individuals in the United States population who did not receive or need any antibiotic during 1954 and, consequently, it can be safely assumed that the waste of drugs was considerable.

The sale of antibiotics is a very large and lucrative business. Since antibiotics are generally dispensed on a physician's prescription, the road to bigger sales leads through the physician's door. He is under great pressure to prescribe the "newest," "best," "broadest" antibiotic preparation, prescribe it for any complaint whatever, quickly, and preferably without worrying too much about specific etiologic diagnosis or proper indication of the drug. The pressure comes from several main sources: (a) In lay magazines and newspapers patients read exaggerated, uncritical, and often misleading claims made for newly discovered drugs. "Scientists announce new potent weapon against colds." "Antibiotic cures and prevents many infections." "New drug saves lives." Most of these accounts are quite meaningless, yet patients proceed to demand the new, marvellous drug from their doctors. The physician may be embarrassed to admit that he knows nothing of this supposed discovery (many doctors find it necessary to read medical news in "Time," "Reader's Digest" and similar media to cope with their patients' pseudo-knowledge), or, he may prefer not to go into lengthy explanations as to why he thinks little of the new drug. It may be simpler and quicker to yield to the patient's insistent demand, and prescribe. This decision may be colored sometimes by the patient's veiled threat to seek medical advice and care elsewhere.

(b) The "medical representative" of the drug company visits the physician frequently, laden with rich gifts of drugs. He is generally a well-briefed salesman of considerable persuasion. He desires to help the doctor, to clarify his ideas regarding a certain drug, and to lead him out of the bewildering jungle of confusing trade names and conflicting claims. He points out convincingly that the antibiotic manufactured by the company he represents is safe, effective, and will serve the doctor well in all circumstances. If he is successful, his soothing talk will make the physician remember a single antibiotic trade name and prescribe it freely. With the availability of a multitude of specific, effective drugs, many physicians are plagued by the question: "Have I really done everything possible for the patient? Have I given him every drug that could possibly benefit him?" The medical representative restores the physician's peace of mind and resolves his conflict. He urges him to prescribe whenever in doubt, reassures him that the drug

he sells usually helps, never harms. Thus, quantities of antibiotics are prescribed for the sake of the physician's psyche rather than the patient's infection.

(c) Drug advertisements, the "siren song of the purveyor of pills" (4) bombard the physician relentlessly and from all sides. His desk and his wastebasket groan under the daily load of letters, pamphlets, and samples that eagerly keep him "informed" on the merits of each preparation. Similar messages greet him from the pages of all medical journals. Advertising revenue is important in defraying publication costs of professional journals, and therefore it is difficult to object to judicious and circumspect advertising for drugs or appliances. One journal page per issue, describing the merits of a given product would suffice to keep the physician adequately informed. This, however, is manifestly old-fashioned. During 1954-55 many medical journals carried in every issue from four to six pages of advertising for a single antibiotic preparation. Whether this advertising promotes sales by appeal or by irritation is difficult to estimate, but it appears that the good taste of the publication is placed in jeopardy.

The pressures on the physician (and his sometimes weak resistance) have been described in some detail because they are among the factors responsible for the enormous abuse of antibiotics. It is a safe guess that not more than 5 to 10 per cent of the hundreds of tons of antibiotics produced every year are employed on proper clinical indication. Does this waste harm anything more than the patient's pocketbook? Does it perhaps produce subtle benefits?

UNTOWARD REACTIONS TO ANTIBIOTICS

A number of review articles have dealt with the various side effects encountered with antibiotics, from the unpleasant to the fatal (5, 6, 7). The most important reactions are caused by hypersensitivity, direct systemic toxicity, and overgrowth by resistant microorganisms. Lowell (8) discussed allergic reactions to sulfonamide and antibiotic drugs. He points out that much of the population has contact with these drugs by chewing gum, sucking on lozenges, self-administered nasal sprays and drops, eating fowl, and drinking milk. Even polio vaccine contains penicillin. "Who can claim NO previous exposure to sulfonamides or penicillin today?" asks this author, and he indicates that any drug can give rise to virtually any type of allergic reaction. The commonest symptoms are fever and skin rashes. Leucopenia, anemia, joint and kidney involvement, and vascular disturbances are fortunately less frequent. Hypersensitivity often cannot be anticipated: a patient might tolerate one course of drug, yet react violently the next time. Skin tests are occasionally useful with penicillin, but not with other drugs.

The most feared reaction is acute anaphylactic shock, which may be rapidly fatal. Probably several hundred cases of anaphylaxis have occurred, most frequently following the injection of penicillin; since the incidence of penicillin hypersensitivity in the general population in the United States is

near 10 per cent. Anaphylactic reactions can also occur following the oral administration of penicillin (9). Any person who has had a penicillin reaction should avoid the drug. Cross reactions between penicillin G and penicillin O occur. If penicillin has to be administered to a person of known hypersensitivity, corticosteroids may sometimes be used to prevent serious reactions (10). In the case of hypersensitivity to streptomycin or *para*-aminosalicylic acid, desensitization has been carried out successfully (11).

The systemic toxicity of most commonly employed antimicrobial drugs is astonishingly low. Penicillin is virtually non-toxic for humans. Daily doses of 60 gm. injected parenterally for several weeks have failed to produce any unpleasant side effects. The toxic effects of ordinary doses of streptomycin for the eighth nerve have been greatly reduced in frequency since it became accepted practice to use a mixture of streptomycin and dihydrostreptomycin. Each component of such mixtures exerts a toxic effect on a different portion of the eighth nerve, yet both have the same antibacterial action. Thus the mixture, for any given antibacterial effect, has only half the toxic action of each constituent.

The direct toxic side effects of the tetracyclines (chlortetracycline, oxytetracycline, tetracycline) are unpleasant rather than serious. A controlled study of the gastro-intestinal side actions, particularly nausea, vomiting, and diarrhea, indicated that 71 per cent of the test group developed these symptoms when receiving oxytetracycline, but 26 per cent developed indistinguishable symptoms with a placebo. However, vaginal itching and burning, pain on urination, and rashes, developed only in persons receiving the active drug (12). Plain tetracycline induces fewer gastro-intestinal reactions than chlor- or oxy-tetracycline, and has therefore found favor with many physicians (13).

When chloramphenicol became available a number of years ago, it was widely employed for a variety of minor complaints, particularly nonbacterial respiratory infections, largely on the premise that it was well tolerated and did no harm, even when its beneficial effect was very dubious. At least eight million individuals are believed to have taken the drug during its heyday. Subsequently, a very few of them developed aplastic anemia and some fatalities ensued. Many physicians became greatly alarmed and refused to use chloramphenicol, even when it was clearly indicated as the drug of choice. More recently the drug has staged a comeback and has been employed effectively in salmonella and staphylococcus infections. A critical study (14) finally established that hematopoietic tissue in man could be depressed by chloramphenicol, but that only large doses (6 to 12 gm. daily for 12 to 35 days) produced this effect. These amounts were far in excess of desirable clinical dosage for optimal antibacterial effects, and the abnormalities of the bone marrow were reversible when drug administration was stopped promptly.

Some drugs possessing significant toxicity have acquired increased usefulness. Because of the restraint with which they have been employed, mi-

crobial resistance to them has emerged only rarely. Thus polymyxin, bacitracin, or neomycin can sometimes cure infections caused by organisms resistant to all other available antibiotics. The toxic properties of these drugs can be minimized and therapy made safe, by proper attention to dosage and method of administration (15).

When a patient responds initially to the administration of an antibiotic, but subsequently the symptoms and signs of infection recur, "superinfection" with resistant organisms is one of the possible explanations. In the large experience of Weinstein, Goldfield & Chang (16) infections during chemotherapy occur in about 2 per cent of patients, usually in the same organ or region of the body as the original disease. Such infections occur most frequently in children under three years of age after four to five days of treatment with one of the so-called "broad-spectrum" antibacterial drugs. The change in the infecting organism can be detected only by frequent bacteriological study. Such "superinfections" are especially dangerous when antibiotics are administered for a disease not tractable by chemotherapy, e.g., most common viral infections. Anatomical and physiological abnormalities in an infected organ system predispose to superinfection. This is demonstrated most dramatically in the urinary tract (17). There, the suppression of an infection in an abnormal urinary tract is almost invariably accompanied by the establishment of other bacteria, resistant to the chemotherapeutic agent administered. Furthermore, an indwelling catheter in a hospitalized patient frequently promotes the establishment of antibiotic-resistant "hospital strains" in the urinary tract. Thus there is an increase of urinary tract infections caused by relatively drug-resistant bacteria, such as coliform bacilli, *Aerobacter*, *Pseudomonas*, and *Proteus* (18).

The suppression of normal flora is undoubtedly the most important feature in the frequently observed superinfections with *Monilia* (*Candida*). Moniliasis (candidiasis) of mucous membranes (oral, vaginal, rectal, perhaps intestinal) occurs frequently during tetracycline treatment, less commonly when other antibiotics are administered. In most individuals with normal host resistance such monilial proliferation during antibiotic therapy causes unpleasant side effects, principally intense anal and vulval itching, or diarrhea, but is not a serious threat. In debilitated patients, on the other hand, who suffer from some chronic degenerative disease, and whose general resistance is impaired, disseminated candidiasis occurs, with lesions in many organs contributing to the patient's death (19). Available antifungal drugs like nystatin or *para*-hydroxybenzoic acid inhibit *Candida* locally upon direct contact, but have no effect in the disseminated disease.

It was known for several years that chlortetracycline could stimulate the growth of *Candida* *in vitro* and *in vivo* (2). Additional experiments indicated that *in vitro* chlortetracycline stimulated growth of *Candida* in concentrations of 5 to 60 $\mu\text{g./ml.}$, but in lower or higher concentrations it resulted in inhibition. The effect was principally a stimulation of early growth rate but not an increase in the total population of yeasts (20). The growth

of *Candida* in broth was enhanced not only by chlortetracycline but also by neomycin or bacitracin (21). After oral administration to mice many antibiotics produced a significant increase in the number of *Candida* organisms in the intestinal flora. This occurred even with drugs which did not stimulate the growth of *Candida in vitro* (22). Thus it is likely that change in microbial flora is an important factor in such "superinfections" by *Candida*, in spite of the disagreement among authors (23).

Reactions due to hypersensitivity or to direct toxicity of antibiotics, superinfections, and resistance (see below), can be charged to the debit side of the indiscriminate large scale administration of antimicrobial drugs. What might be listed on the credit side of this abuse? It is probable that the most highly antibiotic-susceptible bacterial pathogens, e.g., hemolytic streptococci or pneumococci, are occasionally eradicated at the very inception of a disease process, when their activity has not yet led to significant pathologic changes. Thus a certain amount of disease is probably prevented accidentally; how much, and at what cost, is not known. It seems clear, however, that the disadvantages of the large scale indiscriminate administration of antibiotics far outweigh its possible benefits and should be vigorously condemned. The problem of antibiotics used for prophylaxis is discussed below.

RESISTANCE

The problems of microbial resistance, from the standpoint of medical chemotherapy, were reviewed by several authorities (24, 25, 26). Bryson & Demerec (24) discussed mechanisms of the emergence of antibiotic-resistant bacteria. They concluded that once mutations to resistance had occurred in bacterial populations, the over-all gain or loss of resistance in the population depended upon factors of selection. Here the presence or absence of antibiotics played the major, but not exclusive, role. Multiple chemotherapy was the most efficient method for preventing the establishment of resistant strains because of the rarity of double mutants. Among the factors which limit its effectiveness in practice, they list the following: (a) growth of resistant colonies before the onset of antibiotic therapy; (b) possible occurrence of induced multiple mutations; (c) multigenic basis of some types of resistance which leads to increased probability of mutation; (d) increased significance of cross-resistance as new antibiotics are discovered; (e) the possibility that nonspecific resistance will protect bacteria to a slight degree against several antibacterial agents used simultaneously, allowing multiplication, mutation, and selection of more highly resistant forms; (f) the probability of consecutive development of resistance to several agents present simultaneously.

Lepper views the microbial resistance to antibiotics from the standpoint of the treating physician (25). He differentiates the "development" of resistance within the body of a given patient, from the acquisition of resistant mutants in the environment. The first type is exemplified most markedly

by tubercle bacilli where the transmission from one host to another is relatively unimportant (see below). The second type is encountered particularly with staphylococci and, to a lesser degree, with gram-negative bacilli in a hospital environment where the patient often acquires drug-resistant organisms from physicians, nurses, and other patients, and transmission from host to host is the paramount factor. "Development" of resistance by staphylococci is unimportant, except with streptomycin and erythromycin. The following are among the remedies recommended by Lepper (25) to combat the rise in antibiotic-resistant infections: restrict use of all antibiotics to a minimum; treat only for evidence of active infection, not merely for the presence of bacteria in chronic non-tuberculous infections (particularly in the respiratory, urinary, and gastro-intestinal tracts); remedy the anatomic, immunologic, or metabolic defects in the host which predispose to chronic or recurrent infection.

In a truly monumental piece of work (30 pages, 564 references), Finland (26) reviewed the reviews and analyzed many of the clinically pertinent articles on antibiotic-resistant bacteria. A few of the conclusions may be summarized here. Among group A beta-hemolytic streptococci, types 17 and 19 were resistant to sulfonamides, but there was no evidence for the acquisition of resistance to either sulfonamides or antibiotics in any originally sensitive strains of beta-hemolytic streptococci. The same is true for meningococci and for most strains of *Streptococcus viridans*. Many strains of gonococci are resistant to sulfonamides but none to penicillin or other antibiotics. The most troublesome antibiotic-resistant pathogens are staphylococci. Among organisms in which resistance frequently develops within the original host, the tubercle bacillus is perhaps the most important.

A distinct category of antibiotic-resistant microorganisms is that which replaces the original flora suppressed in the course of drug treatment. This occurs during the administration of antibiotics, most frequently in the respiratory, gastro-intestinal, and urinary tracts. The normal microbial flora is harmless and may even maintain homeostasis for the organ system. Its replacement by other organisms may lead to disturbances in function. The organisms most commonly replacing antibiotic-sensitive microorganisms are staphylococci, *Proteus*, *Pseudomonas*, *Aerobacter*, coliform bacilli, various yeasts, and fungi. The same organisms can cause primary hospital-borne ("nosocomial") infections which are observed with increasing frequency.

With regard to biologic and biochemical properties, antibiotic-resistant strains often do not differ significantly from sensitive strains of the same species. Exceptions are penicillin-resistant staphylococci, which regularly produce penicillinase, and isoniazid-resistant tubercle bacilli, which often fail to produce catalase and are avirulent for guinea pigs (26).

Cross-resistance has been most clearly demonstrated against antibiotics having closely related chemical structures, e.g., streptomycin and dihydrostreptomycin; tetracycline, oxytetracycline, and chlortetracycline; erythromycin, carbomycin, and perhaps spiramycin. There are disagreements

among investigators concerning the degree of cross-resistance encountered in some of these groups, particularly the tetracyclines (27). From a practical standpoint, however, it is acceptable to consider that cross-resistance almost complete. Increases in sensitivity to heterologous agents may accompany the development of increased resistance resulting from repeated subcultures in some antibiotics. Those interested in the medical aspects of microbial resistance must consult Finland's massive article in detail. The problems of resistance in staphylococci and in tubercle bacilli form well-defined special cases and are discussed separately below. Penicillin-resistant variants have been found among the antibiotic-sensitive large viruses of the psittacosis-lymphogranuloma venereum group (28). A thousand-fold rise in the penicillin tolerance of virus was observed in the course of passages in the yolk sacs of fertile eggs injected with increasing amounts of penicillin. This rise was maintained upon subsequent passage in the absence of the drug. The drug-resistant variant was more sensitive to chloramphenicol, but less virulent for mice than the parent strain.

STAPHYLOCOCCAL INFECTIONS³

For a number of years it has been observed in hospitals throughout the world that the staphylococci grown from attending personnel and patients tended more and more to be resistant to penicillin and other antibiotics (29). This change, the suppression of drug-sensitive staphylococci and their replacement by drug-resistant ones (30), was clearly a result of the exorbitant use of antibiotics in the hospital. In many hospitals penicillin-resistant staphylococci comprise 60 to 85 per cent of all those cultured. Fortunately this change has not yet occurred outside institutions and hospitals. Of staphylococci isolated from pyogenic lesions or skin of outpatients in London, only 21.5 per cent were resistant to penicillin, and very few to the other available antibiotics. This represents little change from the situation surveyed three years ago (31).

Within hospitals, however, the situation is serious. Of 516 strains isolated from staphylococcal carriers at Bellevue Hospital in New York in 1954, 65 per cent were resistant to tetracyclines, penicillin and streptomycin. Of these resistant organisms, 90 per cent belonged to phage group III pattern, whereas only very few staphylococci cultured from patients on admission fell into this same pattern. Thus staphylococci of the group III phage pattern were evidently acquired in the hospital, more rapidly after treatment with tetracyclines than after administration of penicillin. Resistant "hospital" staphylococci were acquired more slowly in the hospital if the patient received no antibiotic treatment (32).

This rise of the resistant hospital staphylococci has several practical aspects beyond its epidemiological interest. Reporting from the Massachu-

³ The term "staphylococcus" is used here for *Micrococcus pyogenes* var. *albus* or var. *aureus*.

setts Memorial Hospitals, Howe (33) found an appalling rise in wound infections following "clean" surgical operations. In 1949 the rate was 1.99 per cent in clinic patients and 0.63 per cent in private patients. In 1953 the respective figures had risen to 7.22 per cent and 2.25 per cent. The average rate of wound infections in clean surgical cases had nearly quadrupled from 1.09 per cent in 1949 to 3.98 per cent in 1953. Unless it were to be assumed that surgical skill had diminished during those four years (and few practitioners would admit to that), the rise of infections must be attributed to the saturation of the hospital environment with drug-resistant organisms. Similarly the higher rate of infection among clinic than among private patients merely reflects the more extensive contact of clinic patients with doctors, nurses, attendants, and other patients, as sources of resistant organisms. The unnecessary saturation of the hospital environment with antimicrobial drugs is to blame in large measure for the prevalence of drug-resistant staphylococci. In this regard Howe points out that there is doubt about the "wisdom and efficacy of routine prophylactic systemic antibiotic therapy in clean surgery. It is difficult to find scientific justification for its use . . . it will mask and delay the appearance of wound infection but apparently will not prevent it The disadvantages of routine systemic prophylaxis outweigh the possible benefits when applied to clean, uncomplicated surgery in civilian practice." One can only hope that these admonitions will not fall upon deaf ears.

Additional evidence on the unnecessary and possibly harmful excessive use of antibiotics in hospitals was published by Loh & Street (34). During September 1953 a community hospital in New England had 458 patients (excluding delivering mothers and newborns), of which 291 (63.5 per cent) received one or more antibiotics. Of 232 surgical cases treated with antibiotics, 190 (81.9 per cent) received the drugs without evidence of infection at the time. Of 88 medical patients treated with antibiotics, only 9 (10.2 per cent) had no evidence of infection at the time. Thus, the great bulk of antibiotics was given to surgical patients for "prophylaxis" whereas most medical patients received drugs for existing infections.

"The dilemma of hospital infections has been complicated rather than solved by chemotherapy," complains Lowbury (35), in a discussion of cross-infection of wounds and burns with antibiotic-resistant organisms, particularly staphylococci, *Proteus* and *Pseudomonas*. He urges greater emphasis on preventing spread of organisms in hospitals, particularly by the air. Among possible ways to diminish the emergence of resistant bacteria, he recommends economy in the use of antibacterial drugs, particularly avoiding agents which readily induce resistance; use of drug combinations; and "therapia sterilisans magna," i.e., drug therapy which rapidly eradicates infecting organisms rather than merely suppressing them.

Staphylococcal enterocolitis is a special and much-feared complication of surgery in the hospital which continues to receive attention. This illness has been associated with the use of tetracycline drugs for the pre-operative

preparation of the large bowel. When these drugs are administered there is a marked depression of the total number of microorganisms in the bowel flora for a few days, followed by an over-all increase in numbers (36). Commonly *Proteus*, *Aerobacter*, anaerobes, and yeasts account for the rise in bacterial numbers. However, in an environment laden with antibiotic-resistant staphylococci these organisms may implant themselves on the mucous membranes of the bowel temporarily freed from their normal flora. The staphylococci may multiply very rapidly so that pure cultures of these organisms are found in the stools. Some patients have no symptoms; others develop diarrhea, shock, and death from pseudomembranous enterocolitis (37, 38). While antibiotic-resistant staphylococci have been implicated in this disorder, their role has never been entirely clear. Two additional suggestions have been made to explain the pathogenesis of staphylococcal enterocolitis. Surgalla & Dack (39) thought that staphylococci might injure the intestinal mucosa by enterotoxin locally produced in high concentration. McKay *et al.*, (40) demonstrated experimentally that dogs infused with human blood and subjected to surgical trauma went into irreversible shock and developed pseudomembranous enterocolitis histologically almost indistinguishable from the disease in humans. As a pathogenetic mechanism, they postulated stasis of blood in the capillaries of the intestinal mucosa and intravascular clotting. In the human disease staphylococci might contribute to the vascular disturbance. This explanation is of considerable interest because it indicates that staphylococci are only one of many possible associated causes of pseudomembranous enterocolitis. In some hospitals the cases of enterocolitis have been recently associated with pre-operative administration of tetracycline, but, compared to the pre-antibiotic era, there has been no definite over-all increase in the incidence of this complication in surgical patients.

During 1955 a number of hospitals experienced outbreaks of staphylococcus infections in their newborn nurseries. Evidently the organisms spread from the respiratory tract and hands of attendants, the air, and older infants to the skin, respiratory tract, and intestine of newborn infants, and establish themselves there. From the newborn, acting as "enrichment medium," staphylococci reached nursing mothers, producing breast abscesses, and in some instances afflicted other members of the family. Newborn children may continue to harbor the resistant "hospital" staphylococci for many months after discharge from the hospital (41).

Systemic staphylococcus infection with blood stream invasion leads occasionally to endocarditis. This is at present the least tractable form of bacterial endocarditis. Even when all available antibiotics are employed in maximal tolerated dosage, bacteriological cure is obtained only in a small proportion of patients (42). Present cure rates are much below the 54 per cent over-all survival rate among 38 cases of staphylococcal endocarditis seen at Johns Hopkins Hospital during the past 10 years (43).

Knight & Collins (44) estimate the present mortality of all systemic

staphylococcus infections at 50 per cent. They propose that in spite of the marked increase in antibiotic resistance of staphylococci, the present mortality is of the same general magnitude as it was during the early days of penicillin therapy, but lower than in the pre-antibiotic era. These authors feel that the high mortality is attributable to the basic severity of the disease and its frequent occurrence in patients with secondary illnesses (e.g., diabetes, vascular disease, malignancy), rather than to the antibiotic-resistance of the organisms.

Pulmonary inflammation is a common complication of mucoviscidosis (fibrocystic disease) and it is often due to staphylococci. Long-term administration of tetracyclines markedly prolongs the life of children with mucoviscidosis and reduces their complications. The clinical response to antibiotics continues even when the staphylococci are completely resistant to the administered drugs (45). The mechanism of this therapeutic response is not understood.

TUBERCULOSIS

Antibiotics have not dealt as effectively with tuberculosis as with many acute bacterial infections. Nevertheless, the advent of effective anti-tuberculous chemotherapy has brought about radical changes in the outlook for the individual patient and the approach to his treatment. The great decrease in the number of patients resident in tuberculosis hospitals and sanatoria was illustrated most dramatically in 1954 by the closing of Trudeau Sanatorium. This institution was founded in 1894 by Dr. Edward L. Trudeau as the "little red cottage" for treatment of tuberculosis by rest in the healthy air of the Adirondacks, at Saranac Lake. At the time of its founding, the national death rate from tuberculosis was in excess of 200 per 100,000 population. Today the tuberculosis mortality rate in the United States is less than 12 per 100,000 (46).

Hinshaw (47) reviewed editorially the progress in the treatment of tuberculosis during the ten years that had elapsed since he treated the first patients with streptomycin. During these ten years, a 75 per cent reduction in deaths from tuberculosis was accomplished in the United States. Much of this is attributable to improved methods of treatment. The duration of necessary hospital stay was reduced by 50 per cent, a substantial economic gain. Yet, there was only a slight reduction in the prevalence of known active cases.

Isoniazid has been the most important contribution to the therapeutic armamentarium in recent years. Most patients tolerate the drug well and gain weight strikingly early in its administration. One of its troublesome side effects, peripheral neuritis, may occur in 40 per cent of patients, and is due to the excessive excretion of vitamin B₆. This is somewhat proportional to the dose of isoniazid and can be prevented by the administration of vitamin B₆, 300 to 450 mg. daily (48). The pyridoxal-isoniazid antagonism evidently plays little role in the antibacterial effect of the drug.

Studies on the mode of action of isoniazid have yielded the following information. Tubercle bacilli exposed *in vitro* to isoniazid under conditions otherwise favorable for growth, lose their acid-fast staining characteristics. Simultaneously, with a slowing of growth rate, there is a decrease in tetrazolium reduction. Complete loss of acid-fastness is associated with failure of the cells to grow in subculture. In contrast, tubercle bacilli exposed to streptomycin are inhibited in growth and tetrazolium reduction, but their acid-fastness persists. When such organisms are removed from the influence of streptomycin, they resume growth and tetrazolium reduction. Thus under experimental conditions streptomycin appears to be bacteriostatic; isoniazid, bactericidal (49).

Isoniazid labeled with C^{14} is not bound by killed tubercle bacilli and is bound to a greater extent, by tubercle bacilli whose metabolism is "arrested" by suboptimal temperature, streptomycin, or *para*-aminosalicylic acid, than by cells growing at an optimal rate. The relationship between the concentration of isoniazid in the medium and the amount of drug bound by cells can be expressed by Freundlich's equation for physical adsorption. Thus the fixation of isoniazid to susceptible tubercle bacilli appears to be governed by mechanisms different from the fixation of penicillin to organisms susceptible to penicillin (50).

The activity of isoniazid was compared with that of streptomycin on tubercles in a transparent chamber in the rabbit ear (51). Neither of the drugs alone, nor both together could completely destroy tubercle bacilli of the virulent Branch strain in tissues of the rabbit. Even when lesions resolved entirely during treatment, they reappeared when drug administration was stopped. This was true even when treatment had continued for two months after apparent healing. When caseation had occurred, healing was usually slow. The presence of living tubercle bacilli within caseous lesions seemed a more important factor in delaying, or preventing, ingrowth of capillaries, than the physical consistency or chemical constitution of the caseous material. The authors feel that their experimental studies suggest the need for earlier diagnosis, before there is far-advanced caseation, and longer treatment after healing appears to be complete.

As with other anti-tuberculous drugs, the efficacy of isoniazid is limited by the rapid emergence of isoniazid-resistant tubercle bacilli. Perhaps resistance to isoniazid may not detract from its therapeutic effectiveness as much as originally believed (52, 53). Among human tubercle bacilli and their isoniazid-resistant mutants, there appears to be a relationship between degree of drug resistance, catalase activity, and guinea pig virulence: as isoniazid resistance increases, catalase activity and pathogenicity for guinea pigs both decrease. Such mutants remain pathogenic for mice. However, since virulence for guinea pig and for man are believed to be parallel, it appears possible that isoniazid-resistant mutants may be unable to cause progressive tuberculous disease in man. This point is by no means settled.

For the time being it is widely accepted that in all but the most minimal

types of tuberculous disease, it is desirable to delay the emergence of isoniazid resistance of high order by combining this drug with other anti-tuberculous agents, usually streptomycin or *para*-aminosalicylic acid. The precise dosage schedules proposed as "optimal" vary from time to time and from one group of investigators to another (54, 55). It is becoming clear, however, that every individual with tuberculosis diagnosed before the disease is far advanced with extensive caseation and cavitation, and who cooperates in well-supervised treatment, has an excellent chance of prolonged clinical remission and useful life. This is indeed a radical change in outlook from what is was just a few years ago. Even those most severe forms of tuberculosis, miliary dissemination and tuberculous meningitis, have yielded, to some extent, to chemotherapy. With combined isoniazid and streptomycin treatment 95 per cent of cases of miliary tuberculosis without meningitis, and 80 per cent of cases of tuberculosis meningitis, now survive 18 months or more if treatment is started early (55, 56). Better still, meningitis virtually never develops in patients treated with isoniazid and one other drug, whereas it used to be a common and highly fatal complication in the past.

In spite of these successes, a search for additional and better anti-tuberculous drugs continues. The ultimate need is for "eradicated chemotherapy," drugs which are capable of reaching and killing all viable tubercle bacilli in tissues, caseous material, cavities, and fibrous scars. As yet, no such possibility is in sight although some new drugs have appeared. Pyrazinamide is highly effective in suppressing experimental tuberculosis of mice. In combination with isoniazid it may have greater anti-tuberculous activity than other forms of combined therapy presently available (57). Unfortunately pyrazinamide induces a severe side effect, hepatitis, found in 3.5 per cent of individuals treated with full doses. For this reason the drug is unlikely to find wide application. The compound *D*-4-amino-3-isoxazolidone (oxamycin, cycloserine) was discovered simultaneously in several laboratories (58, 59). While it had rather limited anti-tuberculous activity *in vitro* and in mice, it showed some promise in humans (60). Unfortunately there also was significant neurotoxicity inherent in the drug in therapeutically employed doses. Thus these drugs are not yet definitely useful additions to the armamentarium of tuberculosis therapy, but there is little doubt that others, more potent and less toxic, will be found.

But, is drug therapy directed against tubercle bacilli the whole answer to the treatment of such a complex process as tuberculosis? Consideration of the host response; innate resistance; immunity; hypersensitivity; environment, will, for many years, play a decisive role in the over-all planning of management of this disease. A few points in this complex problem may be picked at random. The healing of tuberculous lesions under effective antimicrobial therapy may result in greatly increased dysfunction of lung, due to rapidly progressing fibrosis. Thus, the patient suffers a paradoxical increase in symptoms and disability as he improves from the standpoint of tuberculous infection (61). In persons who have long been infected with tubercle

bacilli but are in balance with their disease, experiencing no symptoms and having no manifestly active tuberculous lesions, the activity of the process may be precipitated by such factors as starvation, gastrectomy, or corticosteroids (62). These problems could be dealt with better if more were known about the host-parasite balance of the infection.

A few years ago, in many parts of the world, primary tuberculosis was considered an inevitable part of growing up. It was believed that primary infection imparted a certain amount of resistance and that individuals who had successfully weathered it in childhood were relatively more resistant to progressive tuberculous lesions in adulthood, than those who lacked this primary experience with the tubercle bacillus. However, the process of primary infection is, in itself, fraught with considerable risk. From it there may result segmental pulmonary lesions and pleural effusion (10 per cent), hematogenous dissemination giving miliary, meningeal, skeletal, renal or other lesions (3 to 6 per cent), and chronic pulmonary tuberculosis (5 per cent), depending on the time of observation. On the average, about 15 per cent of children with primary tuberculous infection develop some complication (63). Might all this be prevented by treating each and every child immediately upon conversion to a positive tuberculin skin reaction? The question is pertinent but the final answer is not available. Grzybowski (63) believes that anti-tuberculous chemotherapy should be reserved for tuberculin test converters less than 2 years or more than 12 years old, selected individuals with positive family history, and any child hospitalized for primary tuberculosis. He would leave untreated the bulk of asymptomatic test converters between 2 and 12 years. Others feel that every person who becomes infected with the tubercle bacillus should receive chemotherapy. This raises the question of whether every early treatment might eradicate the tubercle bacilli promptly, leaving the individual as susceptible as before infection. That this may occur is suggested by the findings of Robinson, *et al.* (64). Some infants without demonstrable x-ray lesions, treated promptly upon conversion to a positive tuberculin test, became tuberculin negative again after isoniazid was administered for eight months. Such individuals might acquire primary infections repeatedly upon exposure. Would this be desirable if the person had to live in an environment from which tubercle bacilli cannot, as yet, be eliminated? Others (65) plead for greatly, perhaps indefinitely, prolonged chemotherapy in persons with established tuberculous lesions. They feel that the chance of relapse following present day therapy is high, even after long periods of inactivity, and that available drugs could be administered for a person's lifetime without great risk.

If all persons infected with tubercle bacilli were to receive currently available drugs, might there not be a great increase in drug-resistant mutants? Would cross infection with such resistant bacilli create a new public health problem? An answer might be attempted by an analogy to the threat of streptomycin-resistant tubercle bacilli (66, 67, 68). Among 1166 patients admitted to Veterans Administration Hospitals, who had no previous

drug therapy for tuberculosis, 30 (2.6 per cent) harbored tubercle bacilli resistant to 10 $\mu\text{g./ml.}$ streptomycin. Of the 26 persons in this group who received chemotherapy, 19 responded favorably to treatment with streptomycin used together with isoniazid or *para*-aminosalicylic acid (66). In another study, 600 cases of previously untreated tuberculosis were diagnosed over a five year period. Only 10 (1.8 per cent) of them were infected with streptomycin-resistant bacilli (67). Thus it appears that the present risk of becoming infected with streptomycin-resistant tubercle bacilli is rather small, and that even such infections can be treated satisfactorily with available methods in most instances. On the other hand, it must be recognized that at least one case of tuberculosis caused by bacilli resistant to both streptomycin and isoniazid has been observed in a previously untreated patient (68), that drug combinations have been employed on a large scale only for a short time, and that resistance can emerge to both members of the therapeutic drug combination over a period of time.

THE FORGOTTEN HOST

Before the advent of effective chemotherapy, most of the physician's effort in the treatment of infectious diseases was directed at establishing conditions believed optimal for recovery of the host: rest, good nutrition and hydration, fresh air, sunlight, and a cheerful atmosphere, in addition to a multitude of tonics and hematinics. In the absence of more specific anti-infective measures it was thought that these forms of treatment were helpful by supporting the natural defenses and recuperative powers of the host. With the introduction of sulfonamides and antibiotics, the emphasis shifted away from such nonspecific support of the host. Several years' extensive use of potent antimicrobial agents, together with exposure to an intensive advertising campaign by pharmaceutical firms, instilled in many physicians the belief that any drug which inhibited microorganisms *in vitro* and was reasonably non-toxic, would, of necessity, cure human infections caused by that microbial species. This misplaced emphasis made physicians forget the role played by the host in infection and resulted in gross distortions of the chemotherapeutic approach. It was forgotten that in the test tube drug and microbe interact directly and uniformly, but that *in vivo*, the situation is complicated by the interaction of both drug and microorganism with the host, as well as with each other (69).

There has been a renaissance of emphasis on the contribution of the host during the past few years. This has been sparked by (a) the intensive study of hormonal effects on host responses; (b) the necessity of observing the modifications in host response induced by ionizing radiation; (c) the biochemical definition of a few of the factors which either promote or limit tissue lesions in infection; (d) the failure of antimicrobial therapy to eradicate certain infectious organisms in the host despite high activity *in vitro*.

Dubos has been the foremost student of the host factors which permit infection to turn into disease (70, 71). He and his associates are attempting to

define the metabolic host factors which determine whether an infectious microorganism introduced into an animal body will fail to grow, or grow and establish a latent infection without symptoms, or result in the production of lesions and disease. Conversely, they have also studied the biochemical disturbances resulting from infection in the host. Anyone interested in this field must consult the studies of Dubos' group in the original. A few of their findings in experimental tuberculosis might be mentioned here. A large variety of factors can inhibit the multiplication of bacilli in tissues. Among them are oxygen deprivation and acidic pH in necrotic foci, high carbon dioxide tension, and accumulation of several organic acids. A direct antibacterial effect may be exerted by lysozyme contained in secretions, or by organic amines in tissues, such as spermine and spermidine (72).

Nutrition has long been thought to influence susceptibility to infection. Dubos *et al.*, found that mice kept on inadequate food intake or limitation of protein intake for long periods were unchanged in their susceptibility to tuberculosis, but if food was abruptly withheld for 30 hours from otherwise normally fed animals, there was a marked increase in tuberculosis susceptibility. Substances which raise the metabolic rate, e.g., dinitrophenol or thyroxine, had a similar infection enhancing effect (73). Vitamin deficiencies varied in their influence on host susceptibility (73a). While the mechanism for the observed change in host behavior is not completely understood in any one instance, it seems highly probable that many such factors enter into the end result obtained with antimicrobial chemotherapy. Nothing in the foregoing, however, can be construed to support the current fad of incorporating vitamin mixtures into capsules of tetracycline antibiotics. The sales appeal of such concoctions is evidently much greater than their justification on rational grounds (73a).

D'Arcy Hart published an excellent and thoughtful review of the role of the host in chemotherapy of human tuberculosis (74). He stressed particularly the influence exerted by the host upon destruction of tubercle bacilli by drugs and the host's ability to clear residual infection after chemotherapy has been discontinued and to heal the organic damage. It is these factors rather than the antibacterial effect of the drugs as such, that determine the clinical outcome. Among the interesting developments in the treatment of tuberculosis are certain chemicals (e.g., surface active agents like triton) which have no antibacterial effect, yet enable host cells to destroy bacilli effectively (74). The fate of tubercle bacilli *in vivo* has been discussed comprehensively by Hobby (75).

Host responses can, at times, be altered by the administration of antibiotics. Jackson & Axelrod (76) found that tetracyclines or chloramphenicol either increased or diminished the susceptibility of mice and chicks embryos to several antibiotic-resistant microorganisms, depending, in part, on time, dose, and route of administration. Stevens (77) and Slanetz (77a) demonstrated that some antibiotics could diminish the antibody production of experimental animals to several antigens. In clinical practice it is well recognized

that the early treatment of many infections with antimicrobial drugs can suppress both the development of measurable antibodies and of active immunity. The first of these results in difficulties of serological diagnosis; the second, in relapses of the disease as soon as suppressive treatment is stopped. That is observed particularly in infections with salmonellae and rickettsiae (69). Chloramphenicol only suppresses growth of salmonellae, and clinical symptoms, but does not eradicate infection. This has been proved by isolating the organism from patients' blood clots separated from the drug-containing serum (77b). Since chloramphenicol in concentrations of 25 $\mu\text{g./ml.}$ or more possesses bactericidal qualities for many salmonellae *in vitro*, an intracellular habitat might be postulated for some of the organisms persisting in the blood in the presence of drug.

One of the well accepted mechanisms whereby certain antibiotics increase the susceptibility of the host to infection is based upon the suppression of normal flora, permitting the multiplication of undesirable organisms. Thus oral administration of streptomycin markedly enhances the susceptibility of mice to infection with streptomycin-resistant salmonellae (78). This phenomenon has also been observed in clinical practice. A patient receiving a tetracycline drug post-operatively developed acute enteritis, due to a tetracycline-resistant *Salmonella*. As soon as the tetracycline was discontinued the disease subsided spontaneously and the normal bowel flora returned (79).

The antimicrobial defenses of the host can be severely impaired by all types of ionizing radiation. Irradiated animals or people often die from bacteremia caused by otherwise normal intestinal organisms. Antimicrobial drugs are not as effective against any one type of microorganism in irradiated as in normal animals, presumably because the contribution of the host defenses toward eradication of the infecting organisms is lacking (80, 81). The bacteremia of irradiated animals is commonly caused by gram-negative bacilli. Even without irradiation, these organisms can cause serious damage to the host. When large numbers of gram-negative bacilli are accidentally injected intravenously into man (through transfusion of bank blood heavily contaminated with pseudomonads or coliforms), the organisms liberate endotoxin rapidly and in large quantity, causing vascular collapse and death. Antibiotic treatment in such instances has only a limited effect, because the bacteria do not multiply in the host body to any great extent (82, 83). Vascular collapse from any cause, e.g., hemorrhage, greatly increases, the host's sensitivity to endotoxin, so that a small number of gram-negative bacilli may be lethal (84), in spite of the presence of anti-bacterial agents.

CORTISONE, CORTICOTROPIN, AND INFECTION

Adrenal steroids profoundly influence a variety of host factors important in antimicrobial defenses. Because cortisone and corticotropin (ACTH) are now widely employed in medical treatment, their role in the activation or treatment of infections has become a highly controversial subject. Some (85)

urge the use of these hormones in many types of fulminating infections and suggest that the risk is small; others (86) speak out strongly against their use in infectious processes, except for a very few limited indications. Valid answers can be developed only slowly through controlled clinical experience. Laboratory experiments all too frequently give the answer one desires by slanting question and methodology. For example, it can be readily shown that cortisone in doses similar to those employed clinically can markedly diminish the therapeutic effectiveness of antibiotics in experimental infections (87). However, this effect applies only to minimal effective amounts of antibiotics, and can be overcome by larger doses. The phenomenon is probably of importance in antibiotic-resistant infections occurring during cortisone therapy. On the other hand, it can be shown that minute doses of cortisone may have limited beneficial effects in laboratory animals with certain experimental infections (88, 89), and can enhance the therapeutic action of antibiotics. It is clear that some corticosteroids decrease resistance to infection but there are great differences between compounds. Hydrocortisone (compound F) and corticosterone (compound B) have similar metabolic effects and differ only by one hydroxyl group; yet, the first decreases resistance to infection and depresses immune responses; the second, in the same doses, fails to do so (90). The relative amount of different corticosteroids produced may determine the genetic resistance of different strains of laboratory animals (90). Tetracyclines can cure mice of streptococcal infection during cortisone therapy only if the animals have been immunized previously against streptococci. In nonimmunized mice the drugs fail to cure. Thus it might be assumed that the defense mechanisms which cortisone impairs are distinct from those which are enhanced in immunized animals (91).

When corticosteroids are administered for prolonged periods to patients for a primary indication (e.g., disseminated lupus erythematosus), antibiotics are often administered to "cover" the infection-enhancing properties of the hormone. This does not always prevent infection but tends to favor antibiotic-resistant organisms. Furthermore, corticosteroids tend to mask the signs of intercurrent infection, suppress fever, and thus make early effective antimicrobial treatment all the more difficult (69, 92).

In a few limited clinical infections corticosteroids are definitely indicated. Those are processes associated with the release of endotoxins (e.g., infections caused by brucellae, meningococci, salmonellae, and rickettsiae), where the patient may die in the first few days, before the antimicrobial drugs had time to exert their effect. In such situations, especially in very "toxic" patients, the administration of corticosteroids may result in sufficient symptomatic improvement to give the antibiotics time for action (69, 93). The anti-endotoxic action of cortisone has also been demonstrated experimentally (94). There may be a similar indication for corticosteroids in occasional surgical problems, such as ruptured appendix, where the patient appears too ill for a major operation but can be greatly improved in a few days by these

hormones, so that a definitive surgical procedure can be performed. Again, one must guard against complications which cortisone tends to obscure (85, 92).

Some authors feel that corticosteroids should never be employed in tuberculosis because of the risk of disseminating the disease (86). Others believe that combined therapy with corticosteroids and antimicrobial drugs is definitely indicated in tuberculosis and especially in tuberculous meningitis (95, 96). Perhaps corticotropin (ACTH) in moderate doses does not possess the harmful action of cortisone, and may be beneficial in tuberculosis. It may modify the inflammatory exudate in the meninges, decrease mechanical block and formation of scar tissue. It may have a "vitalizing," detoxifying effect permitting the patient to survive the first two to three weeks under antimicrobial therapy, and, suppress tuberculous arteritis. Cases of tuberculous meningitis have been reported wherein lives may have been saved by the concomitant use of ACTH and antituberculous drugs (96). On the whole, it seems clear that corticosteroids can have both beneficial and harmful effects. It will take time before we learn to exploit the good and avoid the bad.

PERSISTERS

One of the central problems in the therapy of tuberculosis (75) and many other chronic infectious processes is the inability of available antimicrobial drugs to eradicate all infective organisms. In the course of drug therapy certain organisms become "persisters." This term was originally coined by Bigger (97) to denote staphylococci which survived exposure to high concentrations of penicillin, although their offspring were completely susceptible to penicillin. The temporary drug-resistance of "persisters" can be attributed to their metabolic inactivity induced by the presence of the drug. As Davis put it, persisters are "genotypically drug-sensitive but phenotypically drug-resistant" (98). Present chemotherapeutic methods cannot deal with "persisters" once they have developed, but their development can be avoided or minimized by increasing the speed with which the original bacterial population is killed by drug action (99). The eradication of certain infections by synergistic drugs pairs, and the failure of single drugs to accomplish that end might be based on the more rapid bactericidal rate of the drug combination. The faster the rate of killing, the smaller the chance of "persisters" emerging. "Persisters" may play an important role in a number of chronic infectious processes in which antibacterial drugs have only a temporary suppressive effect, but fail to eradicate the infection. Probable examples are pyelonephritis (100), osteomyelitis, and bacterial endocarditis. In the last-named disease, always fatal unless successfully treated, it is well known that bacteriostatic antibiotics, or sulfonamides are not curative. To cure the illness, drugs must be employed which are rapidly bactericidal for the infecting organism (101). Chloramphenicol, tetracyclines, or erythromycin are virtually never able to accomplish this, and, for that reason, should not be employed alone in the

treatment of bacterial endocarditis. Penicillin is so successful in this disease because the causative organism is frequently *S. viridans*, for which penicillin is virtually always rapidly bactericidal in low concentration. To effect cure it is necessary to maintain bactericidal concentrations of penicillin inside the endocardial vegetations for adequate periods. These concentrations depend on the penetration of the drug and on the bacterial content of the vegetation: the more bacteria are present, the more drug is needed (102). The aim of therapy is the eradication of all viable organisms in the shortest possible time, avoiding "persisters" formation. Combinations of penicillin and streptomycin are often synergistic against streptococci. Therefore, combined treatment with these two drugs is often curative in this disease, and requires a shorter course of therapy than believed advisable with penicillin alone (103).

It is quite possible that "persisters" develop during antimicrobial treatment of many infections. It was demonstrated ten years ago that in experimental infections with organisms highly sensitive to penicillin, treatment with adequate doses rapidly killed the vast majority of bacteria in the host, but not all of them (104). A few always were left over (persisters?) to be dealt with, and eliminated by, the normal defenses of the host. The same problem was recently re-studied in a different experimental model (105). Penicillin-sensitive beta-hemolytic streptococci were incorporated into agar discs which were implanted in the peritoneal cavity of rabbits. When the animals were immediately treated with large amounts of penicillin (while the organisms were multiplying rapidly) the discs could be sterilized. However, when treatment was delayed until the rate of multiplication had reached the stationary phase, penicillin failed to sterilize the agar discs. A few "persisters" survived the drug action and were protected from phagocytic cells by the agar layer. This work helps to explain the frequent failure of short courses of penicillin to eradicate beta-hemolytic streptococci from the throat. Persistent streptococci also can be found in the tonsils of carriers who received penicillin in large doses for a short time prior to tonsillectomy.

There are other clinical infections where inadequate antibiotic therapy causes complications. Soon after the introduction of penicillin, mastoiditis almost disappeared as a complication of middle ear infection because the latter usually was treated adequately. Recently, however, operation for mastoiditis has again become more frequent (106). This can be attributed to inadequate therapy. Some children with middle ear infection receive just enough antibiotic to suppress symptoms (and greatly reduce the bacterial population), but not enough to eradicate all organisms. As a result the infection smolders, spreading gradually into adjacent bone, until operation becomes inevitable.

ANTIMICROBIAL AGENTS FOR PROPHYLAXIS

To the laity, the knowledge that antibiotics act on bacteria implies their ability to prevent any and all infections. That explains the large scale abuse of antimicrobial drugs for unnecessary and unreasonable "prophyl-

axis," chiefly for minor respiratory infections which are viral in nature and not affected by these drugs. In civilian populations the bacterial respiratory infections which could be influenced by antibiotic prophylaxis amount to only 3 per cent of the total number of respiratory illnesses (107). While most "antibiotic prophylaxis" is useless and ill advised, there are some definite indications which have been superbly reviewed by Weinstein (108).

In healthy persons, chemoprophylaxis can be employed rationally against gonorrhea (oral penicillin), meningococcus infection (sulfonamides), and beta-hemolytic streptococcus infection (sulfonamides or penicillin). In certain regions of high endemicity chemoprophylaxis is also in order against bacillary dysentery, plague, and rickettsial diseases.

In certain chronic diseases chemoprophylaxis can be employed rationally. It is definitely indicated in any individual who has suffered an attack of rheumatic fever, because infection with any type of group A hemolytic streptococci may reactivate the process and lead to increased cardiac damage. In persons convalescing from an attack of rheumatic fever, streptococcal infection must be prevented by oral sulfonamides or penicillin [if the patient cooperates well (109)], or by the injection of benzathine penicillin once every three to four weeks (110). In previously normal individuals any streptococcal infection should be promptly eradicated to prevent the development of rheumatic fever (111) although Weinstein *et al.* (112) feel that such treatment may suppress symptoms of rheumatic fever without preventing carditis.

Glomerulonephritis is associated with infection by only a few types of hemolytic streptococci (e.g., types 12, 4). There is some type-specific immunity against streptococci and therefore a person is not likely to become infected with the same nephritogenic streptococcal type a second time. Thus chemoprophylaxis cannot be directed specifically against hemolytic streptococci as in rheumatic fever. Nevertheless, Rammelkamp suggests that patients with chronic nephritis be given antibiotics because exacerbations often follow a variety of infections (113). Obviously, it is important to prevent the spread of known nephritogenic streptococci by administering penicillin to all exposed individuals.

Persons with known lesions of the heart valves might be protected against bacterial endocarditis by some form of chemoprophylaxis at times of known heavy bacteremia, e.g., dental procedures or operations on the respiratory or urinary tract. It is not precisely known, however, what type of chemoprophylaxis is most useful and what degree of efficacy can be expected (114). Patients with chronic disability of the respiratory tract (emphysema, bronchitis), or with chronic congestive failure, can be given small doses of antibiotics during the season of highest incidence of respiratory disorders. Some are high in the praise of this approach (115), but its usefulness is by no means established. In cases of bulbar poliomyelitis with tracheotomy, single antibiotics or combinations failed to maintain sterility of the respiratory tract and, in some instances, favored the establishment and persistence of micro-

organisms (116). Thus antibiotics had no prophylactic value in that situation.

In the field of surgery partial suppression of the bowel flora may help to prevent peritonitis in operations on the lower intestinal tract. Poorly absorbed sulfonamides or antibiotics are preferable to systemically absorbed drugs for this purpose (15). In most elective "clean" surgical procedures antibiotics for "prophylaxis" are definitely not indicated (33).

It is very appealing to administer antibiotics in known viral infections in order to "prevent bacterial complications." Regrettably this claimed benefit is purely fictional in many instances. The following examples might serve as illustration. A controlled study of sulfonamides and antibiotics given to children with uncomplicated respiratory infections revealed no demonstrable benefit (117). Bacterial complications occurred in about 15 per cent of all groups and were uninfluenced by the "chemoprophylactic" agent. Among 168 cases of paralytic poliomyelitis, 16 per cent developed respiratory bacterial infections if no antibiotics were given, whereas this complication occurred in 53 per cent of 63 patients who received antibiotics. In these two groups of patients pneumonia (often due to staphylococci or pseudomonads) developed in 22 per cent with antibiotics but in only six per cent without these drugs (108).

Weinstein (117a) analyzed in detail the effects of the "prophylactic" administration of antibiotics to children with measles. Of 130 patients receiving antibiotics at home and subsequently admitted to the hospital, 30.4 per cent had secondary bacterial disease. Of 298 similar patients hospitalized without previous antibiotics at home, only 15 per cent had such bacterial complications. Among 78 patients who received antibiotics in the hospital (for no specific indication), 11 per cent developed bacterial superinfections, whereas this occurred in only 4.6 per cent of 350 patients not given antibiotics. Weinstein concludes that "antimicrobial agents do not prevent the development of serious bacterial infection during the course of measles, and their use may actually be associated with an increased risk of this complication" (117a). This admonition might be considered by everyone the next time he is tempted to take an antibiotic for the common cold.

NEW DRUGS

The pharmaceutical industry employs much money and highly trained personnel in a large screening program for new antimicrobial drugs. Hundreds of antibiotics are found every year. Most of these are substances previously known and tested. A few are new and sufficiently non-toxic to warrant clinical trial. Unfortunately, many of the agents that pass initial scrutiny and reach the market do not live up to expectations. A case in point was carbomycin. Discovered in 1952 (118), it was given a resounding name which suggested wide usefulness (Magnamycin[®]) and medical journals bristled with advertisements. A few initial reports were favorable (why is it that so often the first papers published on any new drug or method of treatment glow with enthusiasm?), but more painstaking investigations indicated

that the drug could "not be recommended as a useful antibiotic in bacterial infections" (119, 120). Furthermore, there is far-reaching cross-resistance between carbomycin and erythromycin, so that the latter (better) drug could replace the former.

Another curious development in recent years was the simultaneous discovery and announcement of new drugs from several independent laboratories. Thus D-4-amino-isoxazolidinone was isolated, and its structure determined, by three groups of workers who named the agent respectively oxamycin, cycloserine, and PA-94 (58, 59, 121). Similarly, tetracycline was developed simultaneously by several firms (13). Another antibiotic variously called cathomycin, streptonivicin, or cardelmicin was isolated by three independent laboratories as an agent of possible usefulness against staphylococci. Fortunately the Antibiotics Division of the Food and Drug Administration kept a watchful eye on new drugs and could rapidly establish their identity (122).

Newly discovered drugs sometimes give cross-resistance with other, already established antibiotics, thus losing some of their potential usefulness. Spiramycin, an agent active against gram-negative cocci, gave partial cross-resistance with erythromycin in the hands of some investigators (123) but not of others (123a). This drug is now called norobiocin.

At the annual antibiotics symposium sponsored by the Food and Drug Administration, a score or more newly discovered antimicrobial substances are usually announced. Most of these rapidly sink into oblivion. If a new drug is to reach the medical armamentarium it must measure up to the high standards of effectiveness set by already accepted agents. One marvels at the extraordinary stroke of fortune that brought to light penicillin, still the most outstanding agent in its field. The enormous antibiotic screening program certainly has not yielded the variety and quality of drugs that might be anticipated from the scope of the effort.

There appears to be no lack of versatility among fungi for the production of antimicrobial substances. A fine example was given by Florey who reviewed the various antibiotics produced by *Cephalosporium* (124). All resemble penicillin G chemically but have different biological effects. Of great potential practical significance are cephalosporin C which is active against staphylococci and is not destroyed by penicillinase; and cephalosporin N which is effective against gram-negative organisms, particularly salmonellae. It is probably identical with sinnematin B. Regretably its instability presents difficulties of manufacture.

The "new" drug of greatest practical importance to arrive on the American market and advertising page in 1955 was not really new. Phenoxy-methylpenicillin (penicillin V) was discovered, described, and adequately studied by a group in Vienna in 1953 (125).⁴ This drug is more stable than

⁴ Having been forgotten after the initial discovery of Behrens *et al.* [*J. Biol. Chem.*, 175, 793 (1948)]

penicillin G at the acid pH of the stomach and therefore produces higher and more prolonged blood levels following oral administration than other penicillins (125, 126, 127). The antibacterial activity of penicillin V is quantitatively similar to penicillin G. This "new" drug permits more reliable oral administration of penicillin for both prophylaxis and therapy of susceptible bacterial infections.

Bacterial infections of the urinary tract currently present the commonest problem of antimicrobial therapy. The aims, accomplishments, and difficulties in the chemotherapy of urinary tract infections have been expertly summarized by Kass (128). In only 10 per cent of cases of chronic pyelonephritis can the infection be eradicated by antimicrobial drugs. This emphasizes the need for early diagnosis and intensive treatment, preferably with bactericidal drugs or combinations capable of eradicating the infection before it becomes chronic (12, 128). In urinary tract infections treated with antibiotics, drug-resistant bacteria often replace sensitive strains. Urological manipulations or catheters also may introduce resistant bacteria from the hospital environment. Among such resistant bacteria *Proteus*, *Pseudomonas*, and coliform bacilli are prominent. Nitrofurantoin is useful against some of these organisms. This nitrofurantoin derivative has strong antibacterial action if high concentrations come into direct contact with bacteria. It is absorbed after oral administration and is excreted in the urine in sufficiently high concentration to inhibit many antibiotic-resistant bacteria (with the principal exception of *Pseudomonas*). Unfortunately the drug has no antimicrobial effect in blood or tissues and can therefore not eradicate bacteria in the kidney. However, it is a useful suppressive agent in lower urinary tract infections (128, 129, 130, 131).

No important new antifungal drugs have come to light. Stilbamidine was the first effective drug for a systemic mycosis (132). It has been employed successfully in sporadic and epidemic cases of disseminated blastomycosis in adults and children (133). Stilbamidine also appears to be effective in sporotrichosis (134). For the other disseminated mycoses there still exists no satisfactory treatment. Nystatin has slight antifungal activity in experimental coccidioidomycosis of mice (135), but no established usefulness in clinical cases of the disease. Ethylvanillate in heroic doses may be suppressive in certain cases of coccidioidomycosis (136), but has no effect in others. Nystatin is useful in topical therapy of superficial moniliasis but useless in the rare disseminated disease.

The place of established antimicrobial drugs in different disorders was summarized by Garrod (137). The essential chemical characteristics of antibiotics of clinical importance were reviewed by Regna (138). A new series of publications entitled "Antibiotic Monographs" presents the fundamental and clinical facts about certain drugs (13, 15, 139), and the laboratory methods for their assay (140). Jukes assembled all important information about the role of antibiotics in nutrition of man and animals (141).

COMBINATIONS OF ANTIMICROBIAL DRUGS

Pharmaceutical manufacturers followed a rising trend to incorporate combinations of drugs into many dosage forms. Among the justifications for this trend, the following were encountered: (a) to suppress all types of bacteria, without the need of specific diagnosis; (b) to prevent the emergence of resistant variants; (c) to potentiate the action of one drug by another; (d) to prevent the overgrowth of yeasts an antifungal drug is added to the antibacterial drug. All these possibilities, no doubt, apply sometimes. However, in many instances, when drug combinations are employed there is no convincing evidence for their desirability, and unnecessary administration of combinations is one of the important abuses of antibiotics.

Combinations of antimicrobial drugs designed to inhibit a mixed bacterial flora are definitely indicated in surface infections of skin or mucous membranes, or in surgical infections (142). In these situations, more than one microbial species regularly participate in the infectious process which is enhanced by impaired tissue resistance. Such disorders are most likely to be controlled by mixtures of bactericidal antimicrobial drugs.

The problems of synergism and antagonism among antibiotics were fully treated by Jawetz & Gunnison (143), and little additional material has been uncovered since. Antagonism is so strictly limited by time-dose relationships that its occurrence in clinical practice is extremely unlikely. Strom (144) attempted to find antibiotic antagonism in patients suffering from scarlet fever who were treated either with penicillin alone, or with a combination of penicillin and chlortetracycline. There was no difference in the clinical response to the two treatments, but positive cultures for streptococci three weeks after the end of therapy were three times more frequent (20.8 per cent) in the group receiving both antibiotics, than in the group receiving penicillin alone (7 per cent).

Unfortunately, there is no unequivocal or generally accepted definition for antibiotic synergism (2). Many forms of simple additive effects are called **synergism**, and commercial literature freely uses the appeal inherent in the term. Waisbren (145) examined various antibiotic combinations acting on staphylococci and found that any drug pair might give additive, potentiating or inhibitory results. Most surprisingly, he described potentiation between tetracycline and chlortetracycline, drugs virtually identical in their antimicrobial activity. Davis & Sevag (146) proposed a complicated, if unconvincing, mechanism for antibiotic synergism. Singh & Mitchison (147) observed that *para*-aminosalicylic acid acting on tubercle bacilli simultaneously with isoniazid or streptomycin, not only delayed the emergence of resistant variants, but also enhanced the bactericidal rate, possibly representing synergism.

Some of the most definite examples of antibiotic synergism have been encountered in patients who could be cured only by rapid bactericidal action. Examples were bacterial endocarditis, osteomyelitis, meningitis, and

acute pyelonephritis. Laboratory tests for antibiotic synergism must therefore give quantitative information on bactericidal, as well as bacteriostatic effects of drug combinations. Available tests were reviewed and a new method was proposed by Jawetz *et al.* (148) which incorporated several desirable features. It was successfully applied to the selection of antibiotic combinations for difficult therapeutic problems.

Anderson *et al.* (149) tested combinations of erythromycin and fumagillin for synergistic effects in the treatment of amebiasis in children. The clinical and follow-up results were satisfactory, but no true synergism was observed. The drug combination had no advantage over the success (93 per cent cure rate) found with oxytetracycline or fumagillin by others (150). Erythromycin alone also appears to be a satisfactory drug for the treatment of amebiasis (139).

CONCLUSION

"We are not unaware of how much caution is necessary in judging a new remedy. We must beware of hasty conclusions. We have exposed the facts and doctors may be anxious to use this drug. This has not yet reached the point of efficiency we hope for. At any rate let doctors not forget the necessity of a bacteriological diagnosis without which they expose themselves to serious mistakes" (151). In this quotation the word "drug" has been substituted for "serum." The admonition appears as timely today as when it was written, 60 years ago.

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MUTUAL RELATIONSHIPS IN FUNGI^{1,2}

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INTRODUCTION

Associations among the members of microbial communities are many and varied and underlie the balance or equilibrium which is reached between different kinds of organisms comprising these communities. Frequently, microbial associations are so complex and sensitive to slight changes in the environment that it is extremely difficult to unfold them and discover the primary phenomena which contribute to the end result. For example, certain fungi (1, 2, 3) may be antagonistic to each other on one substrate, whereas on other substrates they actually stimulate the growth and parasitic capabilities of one another. In still other environments they apparently live together seemingly unaffected by each other's presence except for spatial and nutritional limitations.

Efforts to single out different relationships between certain microorganisms and disregard the influence of others in a mixed population have been criticized from the standpoint that a change in the biotic environment will modify the relationships between these organisms and not give a true picture of natural associations. However, just as in studies of complicated metabolic pathways where the individual reactions must be isolated and proven to establish a proposed cycle of events, so must the individual relationships between microorganisms be established in order to analyze a complex association of microbes. It is the purpose of this paper to uncover in part some of these complex associations and discuss examples of relationships which influence the growth and reproduction of fungi.

Mutual relationships in fungi are considered here to include all relationships in which taxonomically different fungi are involved and which are beneficial to at least one of the participating fungi. The word *mutual* is somewhat in appropriate; but a relationship which is truly mutualistic, in which each partner benefits on an equal basis, is more a concept than a reality.

In the past a number of terms have been employed to designate relationships between organisms. Some of these terms, such as *symbiosis*, have been used in both a broad and a restricted sense. For example, symbiosis has been restricted by Burkholder (4) to the relationship where two or more species live together with mutual benefit. Caullery (5) uses the word to designate the intimate and constant association of two organisms with mutual relationships assuring them of reciprocal benefits. In contrast, Leach (6) has interpreted

¹ The literature survey for this review was completed in December, 1955.

² Paper No. 921, Miscellaneous Journal Series Article, Minnesota Agricultural Experiment Station.

symbiosis in a broader sense, to include all associations in which dissimilar organisms live together in close spatial relationship without necessarily any benefit or detriment to either party of the association. This is the way in which de Bary (7) originally used the term to designate all degrees of parasitism, commensalism, and mutualism. An excellent discussion and interpretation of de Bary's (7) use of the term symbiosis has been published by Hertig *et al.* (8).

In this review *symbiosis* will be used in a restricted sense, to designate relationships between taxonomically different organisms in which there is a permanent or semi-permanent physical union between the individuals. Relationships which do not involve a union of the participating organisms will be generally termed nonsymbiotic.

SYMBIOTIC RELATIONSHIPS

When dissimilar fungi live together in a relationship which is contiguous, and therefore symbiotic, they may have little or no effect on one another (neutralistic symbiosis); one or both participants may benefit from the relationship without causing any injurious effects on the other (mutualistic symbiosis); or one, which may or may not derive benefit from the relationship, is deleterious to the other (antagonistic symbiosis).

Usually, however, the greater our knowledge about a fungal association, the harder it is to fit it into one of these categories. In most cases, the only conclusion which is justified is that the relationship is contiguous or noncontiguous or both.

The terms antagonistic, neutralistic, and mutualistic symbiosis stem from early studies on lichens, mycorrhizas, and micro-plant and insect relationships (5, 9, 10). The parasitic nature of certain fungi in the consortia of lichens of the family Peltigeraceae was observed by Moreau & Moreau (10), who described in detail the morphological characteristics of these lichens and their components. Frank (9), in studies of mycorrhizal associations, used the term mutualistic symbiosis to designate the relationship between mycorrhizal fungi and plants bearing them.

The classification of symbiotic phenomena by McDougall (11) has been used as a guide in fitting together various relationships discussed in this review; however, this classification has been modified here to include under nonsymbiotic phenomena, those disjunctive relationships which are antibiotic, synergistic, or metabiotic in nature.

Antagonistic symbiosis.—Parasitism, which is synonymous with antagonistic symbiosis, has been viewed by Freeman (12) as an intimate association between a parasite and a host resulting from a tendency of the parasite to utilize the host as a source of food. There is no doubt that gradual and continuous changes occur in these relationships which may range from serious injury to no injury, and then apparently to mutual benefit.

Detailed observations on parasitism of *Rozella cladochytrii*, *Rozella rhizophlyctii*, and *Rozella endochytrii* in other chytrids were recorded by Karling

(13) in studies on the host range, pathogenicity, and development of these species. He found that *R. cladochytrii* parasitizes three species each of *Cladochytrium* and *Nowakowskiella*, but does not attack monocentric rhizidiaceous chytrids or filamentous Oomycetes. In contrast *R. endochytrii* and *R. rhizophlyctis* are apparently limited to *Endochytrium operculatum* and *Rhizophlyctis petersenii*, respectively. In summarizing earlier literature on parasitic chytrids, Karling (13) lists seven species in addition to those already mentioned which are parasitic on other chytrids. These are *Olpidiopsis sphaeritae* on *Sphaerita endogena*, *Pleotrachelus paradoxus* on *Rhizophidium distinctum*, *Tylochytrium dangeardii* on *Saccomyces dangeardii*, *Phlyctochytrium synchytrii* on *Synchytrium endobioticum*, *Rozella polyphagi* on *Polyphagus euglenae*, *Rozella maximum* on *Chytridium polysiphoniae*, and *Rozella* sp. on *Rhizophlyctis rosea*. Karling also points out that all known *Rozella* spp. are parasitic on higher Oomycetes and chytrids, causing local hypertrophy or septation of host hyphae.

Recently, Shen & Siang (14), in studies on aquatic Phycomycetes, noted the parasitic attack of *Rozella irregularis* on *Pythium* sp., and *Olpidiopsis saprolegniae* on vegetative hyphae of *Saprolegnia ferax*. The sexuality and parasitic activities of two species of *Olpidiopsis* parasitic on *Saprolegnia* spp., and one *Olpidiopsis* sp. parasitic on *Aphanomyces laevis*, were studied by Barrett (15). He observed that zoospores of these parasites may encyst on host mycelium and produce infection tubes through which the contents of the zoospores pass into the host. The protoplasm of the invading parasite, indistinguishable from that of the host for a short time, later becomes distinct and eventually forms a zoosporangium from which zoospores escape via exit tubes. Germinated zoospores of *Aphanomyces* sp. infected by *Olpidiopsis luxurians* were frequently observed; however, in no case were infected gemmae or reproductive organs of *Saprolegnia* sp. observed.

The parasitism and host range of *Rozella*-like species in the genus *Rozellopsis* have been discussed by Karling (16) in a synopsis of *Rozella* and *Rozellopsis*. He observed that *Rozellopsis inflata* is parasitic in *Pythium intermedium* and causes marked hypertrophy of host sporangia. Prowse (17), in further studies on *R. inflata*, discovered that zoospores of this chytrid may encyst on and then invade hyphae of *Zoophagus insidians*. The zoospore case remains attached to the host wall after the protoplasm of the parasite enters the host. The parasitic protoplasm is not distinguishable until the formation of a zoosporangium in the host hyphae.

In further studies, Karling (16) found that *Rozellopsis Waterhouseii* is parasitic in *Phytophthora cryptogea* and *Phytophthora mesgaperma*, causing hypertrophy of host sporangia and supporting hyphae; that *Rozellopsis septigena* is parasitic in *Saprolegnia monoica* and *Saprolegnia thureti*, causing septation of host hyphae; and that *Rozellopsis simulans* is parasitic in *Achlya polyandra*, *Achlya racemosa*, *Achlya flagellata*, causing slight hypertrophy and septation of host hyphae.

Person *et al.* (18) found that a *Phlyctochytrium* sp. parasitized the oospores

of *Peronospora tabacina* and was probably the cause of erratic infections of tobacco by *P. tabacina*.

In cultures of various root-rotting Oomycetes, Drechsler (19) found two moniliaceous fungi, *Trinacrium subtile* and *Dactylella spermatophaga*, which invaded and destroyed oospores, conidia, and zoosporangia of these phycomycetous parasites. Because of the absence of protoplasmic degeneration before invasion there was probably no readily diffusible toxin involved. *Pythium ultimum*, *P. debaryanum*, *P. irregulare*, *P. mammillatum*, *P. butleri*, *P. vexans*, *P. complens*, *P. arrhenomanes*, *P. salpingophorum*, *P. paroeandrum*, and *P. oligandrum* were parasitized by *Dactylella spermatophaga* in many cultures started from diseased portions of many host plants obtained in different localities. In dual cultures with other Oomycetes on corn-meal agar, *D. spermatophaga* parasitized oospores of *Pythium acanthicum*, *P. anandrum*, *P. dissotocum*, *P. graminicolum*, *P. helicoides*, *P. mastophorum*, *P. myriotylum*, *P. oedochilum*, *P. palingenes*, *P. periilum*, *P. periplocum*, *P. polymastum*, *P. polytylum*, *P. scleroteichum*, *Phytophthora cactorum*, *Phytophthora megasperma*, and *Aphanomyces euteiches*. Infection hyphae of *D. spermatophaga* penetrated oogonial and oospore walls, forming branched haustoria which disorganized and destroyed the cellular contents (19). The parasitic attack on a *Pythium* sp. by *D. spermatophaga* was observed by Peach (20), in studies of aquatic predacious fungi. Drechsler (19) found that *D. spermatophaga* also attacked and destroyed chlamydospores of *Phytophthora cinnamomi* and *P. parasitica*.

Parasitic interactions between various oomycetous fungi have also been studied by Drechsler (21). He observed that the plant pathogenic Oomycetes, *Pythium ultimum*, *P. debaryanum*, *P. irregulare*, and *P. mammillatum*, when grown in culture, did not develop any hyphal relationships suggestive of parasitism on each other. Yet when these species came in contact with a growing mycelium of *P. oligandrum* in dual cultures, their hyphae, zoosporangia, and oogonia were destructively parasitized by the invading hyphae. Moreover, *Pythiogeton autossytum* and *Pythium myriotylum* were attacked in a similar manner by *P. periplocum*, *P. oligandrum*, and *P. acanthicum*. Hyphae of *P. myriotylum* were often coiled by filaments of the parasitic *Pythium* spp. and were invaded by haustorial threads developing from appressorial-like structures applied to hyphae of *P. myriotylum*. Further extension of these invading hyphae was often checked by septa which delimited the invaded regions from adjacent healthy portions of the mycelium. A similar defensive mechanism was displayed by *P. myriotylum* when it was attacked by *Plectospora myxiandra* or *Aphanomyces cladogamus*. In most cultures, however, parasitized hyphae developed a thickening on the inner side of the wall at places where appressoria had become attached, thus preventing invasion by the parasitic hyphae. *Pythium acanthicum*, *P. periplocum*, and *P. oligandrum*, although destructively parasitic on other pythiaceous fungi, were attacked and destroyed by two isolates of *Aphanomyces cochlioides*, *Aphanomyces cladogamus*, and *Plectospora myxiandra*. Drechsler (22) also

observed that oospores of *Pythium graminicolum* were vigorously parasitized by *Trichothecium arrhenopum*.

The effects of nutrients, temperature, light, hydrogen-ion concentration, and the presence of other fungi on the susceptibility of *Pythium debaryanum*, *Mucor recurvis*, *P. butleri*, *Rhizopus* sp. and *R. arrhizus* to parasitic attacks by *Rhizoctonia solani* was studied by Butler & King (23) and Butler (24). Butler found that *R. solani* parasitizes other fungi by penetrating their aerial hyphae and causing the host protoplasm to coagulate and eventually lyse. The host reaction depended upon the parasitic strain employed and on environmental conditions. Among 54 isolates of *R. solani* tested, 4 myco-parasitic races were distinguished on the basis of differential pathogenicity to *P. debaryanum* and *Rhizopus* sp., whereas 2 races of *R. arrhizus* were characterized on the basis of differential susceptibility to a single isolate of *R. solani*. *R. solani* grown on water agar, fresh peas, or wheat germ was avirulent for *M. recurvis*; however, if grown on autoclaved yellow corn meal, oats, or polished rice it was highly virulent.

At 15°C., *M. recurvis* was immune from attack by *R. solani*; but at higher temperatures, up to 25° to 30°C., the susceptibility of *M. recurvis* or the virulence of *R. solani* increased. If cultures on potato-dextrose agar were incubated under white light from fluorescent tubes (145 to 400 ft. -C.) the attack by *R. solani* on *Rhizopus* sp., *M. recurvis*, *P. debaryanum*, and *P. butleri* was inhibited. Mycelial growth of *R. solani* in cultures exposed to this range of white light intensity was less than in control plates incubated in darkness; however, whether this decrease in mycelial growth directly influenced the parasitic attacks on host fungi is unknown as the pigmentation and growth of aerial mycelium of the host fungi were also influenced by the light. Hydrogen-ion concentrations between pH 5.5 and 7.1 favored infection of *P. debaryanum* and *P. butleri* by *R. solani* whereas *Rhizopus* spp. were most susceptible on media adjusted to pH 6.7 to 7.1.

R. solani was injurious to *M. recurvis* only when its hyphae were in contact with the host mycelium; aqueous extracts and filtrates of cultures of *R. solani* had no visible effects.

The parasitism of *R. solani* for *M. recurvis* was inhibited when these fungi were grown on synthetic media, containing D-xylose, D-galactose, cellobiose, or glycogen as a carbon source. *R. solani* was highly virulent for *M. recurvis* on media containing starch, sucrose, maltose, D-mannose, D-fructose, or D-glucose. Both fungi grew equally well on all of these media.

Fungi such as *Aspergillus fumigatus*, *Aspergillus flavus*, *Penicillium oxalicum*, *Penicillium roqueforti*, and *Chaetomium globosum* inhibited the infection of *M. recurvis* by *R. solani*. Occasionally, defensive reactions of host cells involved the digestion of endoparasitic hyphae or walling-off of invading parasitic hyphae or both. Butler also observed that *Trichoderma lignorum* [syn. *Trichoderma viride* (25)] and *Fusarium lini* parasitized *M. recurvis*.

In studies on damping-off of citrus seedlings, Weindling (26) found that

a strain of *Trichoderma lignorum* parasitized *Phytophthora parasitica*, *Pythium* spp., and *Rhizopus* spp. Parasitic hyphae of *T. lignorum* coiled around aerial hyphae of host fungi or grew along them in straight or wavy paths, disorganizing or coagulating the protoplasm of the parasitized hyphae.

Buller (27) observed that *Pilobolus* spp. growing on horse dung in nature are frequently attacked by parasitic fungi which invade the young sporangio-phores, stop their growth, and prevent the production of sporangia. Among the fungi parasitic on *Pilobolus* spp. were species of *Pleotrachelus*, *Piptocephalis*, *Syncephalis*, *Mortierella*, and *Dimargaris*. Buller (27) has also reviewed the early observations of W. Zopf and P. van Tieghem on fungal parasites of *Pilobolus*. Similarly, Weindling (28) has adequately reviewed the early researches of O. Brefeld on the susceptibility of *Mucor mucedo* to parasitic attacks by *Piptocephalis* sp. and *Penicillium* sp., the observations of A. Blochwitz on the parasitic action of *Aspergillus* spp. and *Penicillium glaucum* on mucoraceous fungi, and the studies of H. Burgeff on the parasitic action of *Parasitella simplex* on *Mucor* sp.

Parasitella simplex is commonly parasitic on other mucors, producing galls which may or may not contain a mixture of the cell contents of the host and parasite. These galls resemble zygospores except for basal outgrowths and storage cells. Satina & Blakeslee (29), studied the effects of 4 races of *Parasitella* on 54 races of *Mucor* representing 9 genera and 17 species, on 21 races of *Absidia caerulea*, and on 9 races of *Absidia glauca*. They found that the parasitic relationships between *Parasitella* and these fungi were apparently not sex limited, as previously thought by Burgeff (30).

A species of *Piptocephalis* was reported by Dobbs (31) to parasitize both plus and minus strains of *Mucor mucedo* and *Mucor hiemalis*. In further studies on the host range and parasitic development of *Piptocephalis xenophila* on ascomycetous and mucoraceous hosts, Dobbs & English (32) observed that *P. xenophila* attacked *M. mucedo*, *M. hiemalis*, *Absidia capillata*, *A. glauca*, and *A. spinosa* less vigorously than ascomycetous hosts. None of the Basidiomycetes tested were parasitized by *P. xenophila*. *Piptocephalis* invaded host hyphae by infection tubes which developed from appressoria. After penetrating the hyphae, the infection tubes branched to form haustoria. It is questionable whether the parasitic attacks of *Piptocephalis* were injurious, since the host mycelia of *Penicillium roqueforti* were not killed and continued to sporulate.

In contrast to the wide host range of *Piptocephalis*, Ayers (33) found that another mucoraceous parasite, *Dispira cornuta*, was limited to mucorine hosts. He determined the host range, parasitic specialization, and mode of infection of single-spore isolates of *D. cornuta* in dual cultures with 34 representatives from the Phycomycetes (Mucorales, Entomophthoraceae, Saprolegniaceae, Pythiaceae, and Acrasiales), 8 Ascomycetes (Endomycetaceae, Phacidiaceae, Dermataceae, and Sphaeriaceae), 13 Basidiomycetes (Ustilaginaceae, Tilletiaceae, Thelephoraceae, Hydnaceae, Polyporaceae, and Agaricaceae), and 21 species belonging to the Fungi Imperfecti (Moniliaceae,

Dematiaceae, Phomaceae, and Melanconiaceae). Representatives of every known family of the order Mucorales were parasitized by *D. cornuta*, but the parasitic activities of this fungus, itself a *Mucor*, were restricted to mucoraceous hosts. Isolates of *D. cornuta* obtained from various sources at different times did not differ physiologically or morphologically. Moreover, from an intensive study of the range of variation in structural features of *D. cornuta* and *Dispira americana* in pure culture and on natural hosts, Ayers concluded that *D. americana*, reported by Thaxter (34) to parasitize *Mucor* spp., is not distinguishable from *D. cornuta*. The mechanism of infection was studied in hanging drop cultures seeded with hyphae and spores of *D. cornuta* and *Sporodinia grandis*. In a peptone medium, germ tubes from spores of *D. cornuta* became septate and branched and formed appressoria at the point of contact with host hyphae. Slender infection threads from the appressoria invaded the host hyphae and then branched to form several finger-like haustoria. These haustoria were never found in sporangiophores, zygospores, or aerial hyphae of the different hosts. Moreover, older hyphae of *Sporodinia grandis* which had become thickened and brownish in color were immune from attack. Whether or not these older hyphae were alive is questionable as the hyphae of *S. grandis* are relatively short lived. *D. cornuta* is a facultative parasite and its parasitism is probably attributable to a contact stimulus rather than a nutritive requirement. Response to this kind of stimulus is common in various plant rusts (35) and mycoparasites (24) and has been recently reviewed by Eide (36).

Ayers (33) observed that *D. cornuta* was not parasitic when growing on prune, oatmeal, cornmeal, or malt agars but highly virulent when grown on beanpod and peptone agars. In further experiments he determined that the parasitism of *D. cornuta* was influenced more by the qualitative composition of the substrate than the amounts of various nutrients such as dextrose or peptone. Apparently the interactions between *D. cornuta* and host hyphae approached a neutralistic relationship, since Ayers (33) does not describe any injurious effects on host fungi. In studies of mycoparasitism of mucoraceous hosts, Raybaud (37) observed that a *Fusarium* sp. isolated from potatoes coiled around and invaded hyphae of *Phycomyces nitens*, *Mucor mucedo*, and *Rhizopus nigricans* in dual cultures growing on laboratory media.

Instances of fungal parasitism of ascomycetous hosts have been frequently reported. Slagg & Fellows (38) investigated the effects of certain soil fungi and their by-products on *Ophiobolus graminis* and found that in dual cultures on potato-dextrose agar, the hyphae of *Ophiobolus* were penetrated and killed by those of *Trichoderma*. In other studies Weindling (26) observed the destructive attacks of *T. lignorum* on aerial hyphae of *Sclerotium rolfsii*, while Backus & Stowell (39) found that stromata of *Xylaria oxyacanthae* developed in a natural habitat or in cultures were parasitized by *Fusidium parasiticum*. Koch (40) observed that *Cephalothecium roseum* (syn. *Trichothecium roseum*) and a *Coniothyrium* sp. parasitized the stromata of *Dispira morbosum* on plums and cherries destroying mature and immature

perithecia. Dobbs & English (32) discovered that *Piptocephalis xenophila* vigorously parasitized *Byssochlamys fulva*, *Neurospora tetrasperma*, and *Venturia inaequalis*, in addition to certain Phycomycetes, and several members of the Fungi Imperfecti.

In studies on ergot poisoning in cattle, Simpson & West (41) identified two hyperparasites of *Claviceps paspali* on *Paspalum* spp. During wet weather the sphacelial stage of *C. paspali* was parasitized by *Fusarium* sp. which produced bright orange growth over infected spikelets, whereas a *Cerebella* sp. attacked the ergot and produced a black, irregular mass of fungus growth. The combined action of these parasites prevented the formation of the poisonous stage of *C. paspali*. Greene (42), in a summary of parasitic fungi of Wisconsin, refers to the parasitism of *Phoma parasitica* on *Taphrina mirabilis*.

The common parasite of powdery mildews, *Cicinobolus cesatii* (syn. *Ampelomyces quisqualis*) is a classic example of a fungus living parasitically upon another parasite. Griffiths (43) reported the association of *C. cesatii* and *Erysiphe cichoracearum* on *Grindelia squarrosa* and found that *Cicinobolus* prevented the formation of perithecia by *Erysiphe* and also destroyed the mycelium of *Erysiphe* on lower leaves; however, perithecia were produced in abundance by *Erysiphe* on the upper and younger leaves, and in these perithecia, pycnidia were produced by *Cicinobolus*. Yarwood (44) has emphasized the importance of the pycnidial stage of *C. cesatii* on dead clover and cucumber leaves, which were previously infected with powdery mildew and *Cicinobolus*, as a means for overwintering of *Cicinobolus* in nature.

In further studies, Yarwood (45) isolated *C. cesatii* from *Erysiphe polygoni* on red clover, *Microsphaera alni vacinii* on catalpa, *Sphaerotheca pannosa* on rose, *E. cichoracearum* on zinnia and *Ambrosia artemisiifolia*, *M. alni* on *Lonicera* sp., and from an unidentified mildew of *Plantago major*. Comparisons of cultural characteristics of isolates of *C. cesatii* from red clover mildew, catalpa, rose, zinnia, and ambrosia revealed that these isolates were culturally different from each other. Yarwood (45) also reported almost complete control of clover mildew by *C. cesatii* in experimental plots of red clover.

Emmons (46) has reviewed the early literature on the parasitic activities of *C. cesatii* and has studied in detail host-parasite interactions between *Cicinobolus* and *E. cichoracearum* on *Helianthus tuberosus*. He observed that invading hyphae of *C. cesatii* pass through the walls and into the protoplasm of the host. Initially, cells of the invading hypha are long and narrow; however, they become short and thick by growth and septation and the walls thicken. Cells of *E. cichoracearum* evidently are not killed in advance of the invading hyphae, as the protoplasm of invaded cells shrinks and becomes more granular only after invasion. The parasitized cells soon die, and the invading hyphae grow into other portions of the host or become saprophytic on the remains of dead cells. No obvious defensive mechanism was found once the cells of *E. cichoracearum* were invaded; however, mature conidia and nearly mature ascocarps were apparently immune from attack by *C. cesatii*.

Eventually the *C. cesatii* hyphae invade the epidermal cells and then the intercellular spaces of leaves of the host of the mildew, *H. tuberosus*. Pycnidia of *C. cesatii* commonly develop in the conidiospores, ascocarps, or mycelium of the host.

Basidiomycetous fungi, particularly the rusts, have been frequently noted as hosts of other fungal parasites. In early studies of asparagus rust, Halsted (47) and Smith (48) observed the hyperparasite, *Darluca filum*, growing on uredia of *Puccinia asparagi*. The parasitism of *Darluca* on hemlock rust was studied by Adams (49) who found that the mycelium of *D. filum* ramified throughout the pycnidial and aecial sori of *Peridermium peckii*, destroying the spores and disorganizing pycnia and aecia primordia. Keener (50) discovered biological specialization within the species *D. filum* on 16 macrocyclic and 3 microcyclic rusts. In greenhouse tests, isolates of *Darluca* were separated into 11 distinct groups. Susceptibility of the various rusts differed greatly: *Puccinia sorghi* was attacked by all isolates of *D. filum* whereas *Puccinia poculiformis* was immune to all. The isolates of *Darluca* also differed in their virulence on the 19 differential rust hosts, but no relationship was found between the rust genera from which the isolates of *Darluca* were obtained and the genera on which they were parasitic. Pycnidia of *D. filum* were often embedded in the spore masses of the rust sori and were not visible macroscopically.

Recently, Petrak (51) observed *D. filum* parasitizing *Uredo ravennae* on *Erianthus ravennae*. In other studies Fedorinchik (52) isolated *D. filum* from mycelium and rust pustules of *Puccinia triticea* on wheat. He grew *Darluca* on sterilized pieces of carrot and potato and also on oats and corn seed to obtain inoculum for pathogenicity studies. The cultures of *D. filum* were kept at 18° and 24°C. from one and a half to two months, at which time mass fructification occurred. Fedorinchik noted that when *Darluca* and *Puccinia* spores were inoculated simultaneously on wheat plants, the rust was completely destroyed in the mycelial stage.

The host range of *D. filum* has been appraised by McAlpine (53) who stated in 1906 that *Darluca* had been found on 24 per cent of the species of *Puccinia* and on species of many other rust genera.

Other parasites of rust fungi were reported by Halsted (47) and Smith (48) who observed *Tubercularia persicina* growing upon the cluster-cup form of the asparagus rust. Smith (48) also reported that a species of *Cladosporium* invaded and killed urediospores of *Puccinia asparagi* on rusted plants. The association of *Cladosporium aecidiicola* and *T. persicina* with *Puccinia conospicua* on *Helenium hoopesii* was studied by Keener (54). In notes on parasitic fungi of Wisconsin, Greene (42) reported the parasitic attacks of *Coniothyrium* sp. and *Phyllosticta* sp. on separate telia of *Puccinia anemones-virginianae*; and also the occurrence of *T. persicina* on the microcyclic rust *Puccinia physalidis*, on *Physalis heterophylla* and on *Physalis virginiana*.

Mielke (55) studied the parasitism of *Tubercularina maxima* on pycnia and aecia of *Cronartium ribicola* in western North America and found that

pycnial fluid is apparently the principal substratum for the growth and development of *Tuberculina*. Pycnia are attacked throughout the period of their production, and a pycnial zone attacked one year usually fails to produce aecia the next season. The aecia which do develop are parasitized and production of aeciospores is reduced.

Indications that *T. maxima* also parasitizes the bark well below the rust fructifications on the surface were found when *Tuberculina* sporulated profusely on cut surfaces for at least one month after portions of the infected bark of a stem canker were shaved off. Within the author's knowledge, there were no reports of *T. maxima* on the uredial and telial stages of white pine blister rust on *Ribes* spp. Hubert (56) reviewed the early literature on this fungus and its parasitic habit on six rust species. He emphasized the lack of information on the nutrient requirements of *Tuberculina* and also the difficulties of inoculating rust effectively with its spores for biological control of *C. ribicola*.

In other studies Wollenweber (57) described the observations of L. N. Goodding on the destructive parasitism of *Fusarium bactridioides* on *C. ribicola*, *Cronartium harknessii*, and *Cronartium filamentosum*. Levine et al. (58) noted a *Trichoderma* sp. which overgrew pustules of stem rust on cereals and infected urediospores through the germ pores. Ciferri (59) reported that *Monosporium uredinicolum* parasitized *Coleosporium ipomolae*, the rust of sweet potatoes.

In addition to the rusts, many other Basidiomycetes have been reported as hosts to fungal associates. *Acontium ustilaginicola* on *Ustilago levis* (syn. *U. kollerii*) and *Hypomyces inaequalis* on *Amanita rubescens* were observed by Dickson (60) to destructively parasitize their respective hosts. The suppression of gill formation in fruiting bodies of *Lactarius piperatus* caused by parasitic attacks of *Hypomyces lactifluorum*, which produced many perithecia on the smooth underside of the pileus, was observed by Buller (61). Parasitized fruit bodies of *L. piperatus* were rendered sterile and produced no basidiospores; yet these fruit bodies, even though attacked before they emerged from the ground, were frequently as large as unparasitized fruit bodies. When full grown, instead of emitting a cloud of basidiospores, they emitted clouds of ascospores.

Buller (62) gives an extensive list of nonbasidiomycetous fungi which have been reported to parasitize other fungi, together with his observations and those of others on Basidiomycetous fungi which parasitize other Basidiomycetes. Moreover, he describes the parasitic activities of seven Hymenomycetes which vegetate in the fleshy fruit bodies of other agarics: *Boletus parasiticus* on *Scleroderma vulgare*; *Leptonia parasitica* on *Cantharellus cibarius*; *Claudopus subdepluens* on *Polyporus perennis*; *Volvaria loveiana* on *Clitocybe nebularis*; *Nyctalis asterophora* on *Russula nigricans*; *Nyctalis parasitica* on *Lactarius vellereus*; and *Stropharia epimyces* on *Coprinus comatus*. In the case of *S. epimyces*, the parasitized hosts never elongated their

stipes or opened their pilei although they produced spores. Fruit bodies of *S. epimyces* developed on stunted fruit bodies of the host.

The relationships of *Trichoderma* spp. with *Armillaria mellea* and *Polyporus schweinitzii* were investigated by Aytoun (63). He observed that colonies of 10 isolates of *Trichoderma* spp., when grown on malt agar at pH 3.4, invaded colonies of two strains of *Armillaria mellea*, coiling around and penetrating the *Armillaria* hyphae. After contact with the *Trichoderma* hyphae for at least 24 hr., the protoplasm in the hyphae of *A. mellea* began to disintegrate, followed by the collapse of the hyphal wall. Failure of parasitized colonies of *A. mellea* to yield subcultures indicated that they were killed. At pH 5.1, the parasitic attack on *A. mellea* was less severe than on a more acidic medium and at pH 7.0, parasitism was not observed. An isolate of *Polyporus schweinitzii* was parasitized by the *Trichoderma* isolates on malt agar adjusted to pH 5.1; at pH 7.0 no parasitic interactions occurred.

Bliss (64) found that *T. viride* overran and killed *A. mellea* when the two were planted on the same agar plate and that *Armillaria* could not be recovered from mixed cultures. Similar observations by Darley & Wilbur (65) indicated that *T. viride* invaded and killed *A. mellea* in soils fumigated with sublethal dosages of carbon disulfide.

Occasionally, epidemics resulting in destruction of the spawn and mushrooms occur in cultivated agarics. Suminae & Nakajima (66) noted that spawn and seed cultures of the Japanese edible mushroom, *Cortinellus edodes*, were frequently parasitized by two strains of *T. lignorum*. The attack of *Mycogone perniciosa* on *Psalliota hortensis* f. *albida* in a cultivated mushroom bed near Copenhagen was described by Treschow (67), who reported that 85 per cent of the mushrooms were destroyed. Bitner (68) found *Hypomyces ochraceus* on *Russula* sp., *H. chrysospermus* on *Boletus* spp., *H. linkii* on species related to *Amantia* and *Lepiota*, and *H. lateritius* on *Lactarius* spp. The common mushroom parasite, *Sporodinia grandis* has been reported by Greene (42) on *Lepiota procera* and *Amantia peckiana*.

The wide distribution and host range of the soil inhabiting *Rhizoctonia solani*, which is pathogenic for both fungi and green plants, emphasizes the versatility of this fungus both as a saprophyte and a parasite. Yet the ability of *R. solani* to adapt itself to many kinds of substrates, both living and dead, and to grow and persist under a wide range of conditions does not imply that this fungus is always a good competitor. Boosalis (69), tested the pathogenicity of an isolate of *Penicillium* sp. against 25 isolates of *R. solani*. He found that the coiling hyphae of *Penicillium* infected and killed the mycelium of the host. When grown in a sand-cornmeal medium or potato-dextrose agar (PDA) containing 20 gm. dextrose/l., *R. solani* was severely parasitized; however, when grown on PDA containing 10 gm. dextrose/l., the parasitic activity of the *Penicillium* sp. on *R. solani* was negligible. Damping-off of pea seedlings was greatly reduced in pots containing sterile or nonsterile field soil heavily infested with the *Penicillium* sp.

and *R. solani*. In the absence of the *Penicillium* in control pots, *R. solani* caused 50 per cent of the plants to damp-off, whereas the isolate of *Penicillium* was not pathogenic to peas.

Warren (70) noticed a conspicuous interaction between *Papulospora* sp. and *R. solani*, growing on potato-dextrose, prune, carrot, lima bean, or corn-meal agar. Along the line of juncture of the colonies, a heavy band of mycelium developed in which hyphae of *Papulospora* overgrew, encoiled, and penetrated hyphae of *R. solani*, causing the destruction of host protoplasm. By means of buried slides, Warren found that *Papulospora* parasitized *R. solani* in the same manner in soil. In pots tests *Papulospora* was effective in reducing the amount of sugar beet black rot caused by *R. solani*. Slide cultures indicated that no deleterious effects occurred unless the hyphae of the fungi were in contact. According to Weindling (71), however, when *R. solani* was parasitized on culture media by *Acrostalagmus* spp. *Aspergillus niger*, *Penicillium* spp. *Fusarium lateritium*, *Botrytis cinerea*, and *Verticillium* spp. toxins produced by these fungi were evidently responsible for killing or preventing growth of *R. solani*, both when host and parasite were in actual contact and when they were some distance apart.

In studies on damping-off of citrus seedlings, Weindling (26) found that hyphae of *Trichoderma lignorum* destructively parasitized aerial hyphae of *R. solani*. Occasionally, when planted opposite *Rhizoctonia* on an agar medium, *Trichoderma* did not coil around hyphae of *R. solani*; however, direct attack was induced by planting *Rhizoctonia* on a suspension of *Trichoderma* spores on agar (72). The effectiveness of a lethal toxin produced by *T. lignorum* increased when *Trichoderma* coiled around hyphae of *R. solani* (73). The maximal amount of this toxin was produced, and the strongest parasitic action occurred, two days after germination of the spores of *T. lignorum*. On alkaline media the virulence of *Trichoderma* was attenuated. Additional observations were made by Butler (24) on the parasitism of *T. lignorum* and *Fusarium lini* on *R. solani*. In dual cultures on potato-dextrose agar, *T. lignorum* invaded and killed the mycelium of *Phymatotrichum omnivorum* (74, 75), whereas *Rhizotrichum griseo-roseum* parasitized and killed colonies of *Cercospora capsici* (76).

A species of *Piptocephalis* was reported by Dobbs (31) to parasitize *Aspergillus niger*, *Penicillium roqueforti*, *P. notatum*, *P. pfefferianum*, *P. glabrum*, and several other *Penicillium* spp. Continuing these studies, Dobbs & English (32) found that *Piptocephalis xenophila* parasitized *Cephalosporium* sp., *Diplodinia* sp., *Aspergillus repens*, *Penicillium waksmani*, *P. frequentans*, and *P. camemberti*.

It is interesting to note that this mucorine parasite attacks mucoraceous hosts less vigorously than hosts belonging to the Ascomycetes and Fungi Imperfecti. The limitation of host specificity of certain mucors to mucoraceous hosts has been considered by some workers as an indication of relationship. However, the researches of Dobbs & English (32) and Satina &

Blakeslee (29), indicate that the parasitic tendencies of fungi are not particularly limited by taxonomic groupings.

Neutralistic symbiosis.—Examples of relationships among fungi in which fungal partners live together in intimate association without obvious benefit or harm from their relationship are not readily apparent and probably do not exist in the strict sense. Yet there are several examples which might fit this category.

Parasitic interactions between various Oomycetes have been studied by Drechsler (21). He noted that when a growing mycelium of *Phythium ostracodes* encountered a growing mycelium of *P. acanthicum*, *P. periplocum*, or *P. oligandrum* in Petri dish culture, both continued to advance without a macroscopic change of their circular outlines. On microscopic examination, only a few hyphae of *P. ostracodes* were encircled; however, an increase in the moisture content of the agar medium caused a marked increase in the number of hyphae in the zone of encounter which were enveloped in the hyphal ramifications of *P. acanthicum*, *P. periplocum*, or *P. oligandrum*. However elaborately the hyphae of *P. ostracodes* were encircled by any one of these three parasitic species, no injury or adverse effect from this relationship was found.

According to Røed (77) and Ekstrand (78), *Sclerotinia trifoliorum*, which is a parasite of clover and other legumes, was invariably associated with *Mitrula sclerotiorum* in their cultures. Both are found in clover seed and attack the upper parts of the plants in hot weather. In repeated experiments, sclerotia of *S. trifoliorum* produced apothecia of both species.

Mutualistic symbiosis.—To decide what is mutualistic, neutralistic, or antagonistic in intimate relationships between fungi is often quite arbitrary and depends upon the measure used to evaluate these associations. Thus, a relationship classed as neutralistic from a morphological viewpoint may actually be antagonistic in regard to physiological processes. Several fungal associations have been discovered, however, which apparently fit the concept of mutualistic symbiosis.

Following inoculations of corn seedlings with cell suspensions from certain pairings of haploid lines of *Ustilago zeae* and *Sphacelotheca reiliana*, Rowell & DeVay (79) noted a mutualistic relationship between these smut fungi which resulted in the formation of smut galls. Inoculations with single lines of either fungus failed to cause gall development. Both fungi were recovered from gall tissue and produced similar pathological effects in corn when paired in subsequent tests. Monosporial isolates from chlamydospores obtained from galls of eight different pairings were identical to one another and to the paired line of *U. zeae* in cultural characters, sex factors, and in reaction with various lines of *S. reiliana*. Moreover, the chlamydospores produced in these galls were similar in size, echination, and germination to typical chlamydospores of *U. zeae*. No evidence of hybridization or intracellular associations of nuclei of the different smut lines was found; nevertheless

pathogenic compatibilities of the lines of *U. zae* and *S. reiliana* were apparently associated with certain sex factors and, at least in the early stages of gall initiation, may have been dependent on an intimate association of the cells of the smut lines.

The importance of heterocaryosis as a basis for adaptation in wild fungi to various environments and host plants has been emphasized by Jinks (80), Nelson *et al.* (81, 82), and Christensen & DeVay (83). According to Jinks (80), the association in somatic cells of nuclei of unlike genetic constitution may enable a heterocaryon to survive and grow in an environment unfavorable to homocaryons. In studies on *Penicillium cyclopium* Jinks (80) found that heterocaryosis functioned as a system of limited somatic variation and adaptation well suited to the needs of a fungus living on various and often changing substrates. Recently Nelson & Wilcoxson (81) and Nelson *et al.* (82) in studies of heterocaryosis in *Puccinia graminis tritici*, found that through fusions between hyphae from different races and biotypes, temporary associations of genetically different nuclei occurred which greatly increased the parasitic abilities of the hyphae that contained these nuclei. The creation of a biotype, referred to as the Khapli heterocaryon, which was more virulent than either of the parental biotypes on Khapli emmer and durum wheats occurred when wheat was inoculated with a mixture of urediospores of races 38 and 56 of *P. graminis tritici*. Subsequent pathogenicity tests revealed a marked instability of the Khapli heterocaryon; its rate and degree of dissociation to the parental biotypes of the original races 38 and 56 and also to at least two new races were determined. The instability of the multinucleate condition in urediospores of the Khapli heterocaryon appeared to be associated with decreased virulence of successive uredial generations. The per cent of urediospores of this heterocaryon with three to four nuclei was considerably higher in early generations; however in later uredial generations the percentage of binucleate urediospores of races 38 and 56 and two additional races increased.

The knowledge of symbiotic associations, like those found by Nelson *et al.* (81, 82) between different races of rust fungi, which result in the production of pathogens more virulent than parental types, is necessary in making true evaluations of the pathogenic potentialities of parasitic fungi in nature.

In reviewing various symbiotic relationships between fungi it must be borne in mind that these phenomena are not static and that they may appear to fit one category at the time of observation, but that they may be shifting imperceptibly into other relationships. Furthermore, these studies have been largely concerned with obvious behaviorisms, which may not reflect the changes in metabolic processes which underlie and are influenced by these cellular associations. For example, speculations that haustoria are feeding organs of some parasites may be misleading; it is just as probable that these modified hyphae in some instances act as a source of physical and chemical stimuli to the host cell and cause no deleterious effect (32). Moreover, in some cases infective hyphae may modify the cellular metabolism of

the host cells so that the resultant changes cause the death and digestion of the parasitic hyphae (24).

NONSYMBIOTIC RELATIONSHIPS

The differentiation of microbial relationships such as antibiosis, metabiosis, or synergism from symbiotic phenomena is dependent upon the presence or absence of a contiguous relationship between the participating organisms. Ecological associations of microorganisms which do not involve an actual union of the associates with each other, have thus been classed as nonsymbiotic. Since de Bary (7) first used the term symbiosis, its meaning, like those of many other scientific terms, has undergone an evolution in an attempt to categorize newly discovered facts without inventing new terms or designations. Thus, the universal acceptance of the term symbiosis emphasizes the importance of restricting its meaning rather than broadening it to include phenomena already designated by other terms.

In the past a series of excellent review papers have dealt with the voluminous literature on microbial interactions, particularly antagonistic phenomena, and the exploitation of certain of these relationships for the control of plant diseases (84 to 97). No attempt has been made to review this subject here. However, a number of excellent articles on metabiotic and synergistic relationships have appeared and deserve mention.

Metabiosis.—Metabiotic phenomena or microbial successions on different substrates probably indicate more clearly than any other kind of relationship the marked influence an environment can exert on the growth and development of different organisms, particularly fungi. The occurrence of fungal successions on various substrates has often been noted, although the physiochemical changes in a substrate that favor certain organisms are largely unknown.

Garrett (98) has emphasized the technical difficulties of recognizing the occurrence of an ordered succession of soil microorganisms on a substrate constituting a micro-habitat within the soil. However, the study of soil fungi as ecological groups has been suggested by Garrett (99) as a convenient approach to the investigation of the relationships between these fungi and their habitats. The delimitation of an ecological group of soil fungi is based primarily on the ability of such a group to function as pioneer colonizers of a particular substrate. Thus, the "sugar fungi," represented mainly by *Phycomycetes* are typically found as the first colonizers of injured or dead plant tissues in the soil. Fungi having the ability to decompose cellulose usually follow and most frequently comprise *Ascomycetes* and *Fungi Imperfecti*. Finally, the shortage of immediately available nutrients caused by the sugar fungi and cellulose decomposers probably limits further invasion of these substrates mainly to fungi which have a rhizomorphic growth habit and which can decompose lignin. Most lignin-decomposing species are to be found among the higher *Basidiomycetes*. Similar fungal successions are found on dung.

Tubaki (100), in studies on coprophilous fungi belonging to the Hyphomycetes, noted the succession of fungi on dung collected from various sources and places in Japan. Dungs from herbivorous animals such as hares, deer and antelopes, as well as dungs from lions, birds, and reptiles, were placed on sterilized and moistened filter paper and kept at 25°C. in Petri dishes. At first, Phycomycetes (*Pilobolus*, *Mucor*, *Rhizopus*, *Absidia*, *Thamnidium*, *Piptocephalis*, *Cincinella*, and *Syncephalis*) developed abundantly, followed in succession by Ascomycetes (*Sordaria*, *Pleurage*, *Ascobolus*, *Ascodesmus*, and *Peziza*) and Basidiomycetous fungi (*Coprinus*, *Panaeolus* and *Psilocybe*). In most cases, members of Hyphomycetes developed simultaneously with all the above mentioned groups or appeared abundantly following them. Forty species of Hyphomycetes were isolated by using malt or horse dung agars as culture media. Characteristic successions of fungi, as yet to be discovered, undoubtedly occur on many different substrates and offer a fascinating area for further research. Cook (101) noted that successions on living tissues are usually begun by parasites, which are then followed by common saprophytic fungi and bacteria. On apples parasitized by *Glomerella rufomaculans*, he observed the successive growth of *Rhizopus nigricans*, and later *Penicillium* sp. Tresner *et al.* (102) made a critical study of the relation of soil microfungi to the hardwood forest continuum in southern Wisconsin. They determined the influence of seasonal changes on the frequencies of the most commonly occurring microfungi and also the vertical and horizontal distribution of these organisms in the soil. The areas sampled were precisely defined ecologically and constituted a vegetational continuum which was composed of a continuously shifting series of combinations of tree species in a definite sequence. The more pioneer species were grouped at one extremity whereas the more climax species were placed at the other. The soil microfungi were characterized according to the position in the continuum in which they reached their maximum frequency. Thus, *Penicillium janthinellum*, *Mucor ramannianus*, and *Oospora sulphurea* were "pioneer" species; *P. granulatum* was an "intermediate type"; and *Spicaria violaceae* and *P. nigricans* were typical "climax" species. Moreover, a steady increase in the frequency of *Penicillium* spp. in the soil from the pioneer to the climax forests was found, whereas the opposite was true for the Mucorales.

In studies on other soil fungi, Buchholtz & Meredith (103) noted that *Pythium debaryanum* was most virulent on sugar beet seedlings during the 15-day period after planting, whereas infection of the seedlings by *Aphanomyces cochlidioides* began following the period in which *Pythium* was most active. Similar observations were made by Ho (104), who determined the succession of soil-borne pathogens on corn seed and on seedlings in the field in three successive years. He found that *P. debaryanum* predominated on the seeds in the first isolation cultures. Later the percentage of *Fusarium* spp. and *P. graminicola* greatly increased on the roots and eventually replaced *P. debaryanum*. Various other fungi were associated simultaneously with these pathogens.

The sequence of fungi developing on wheat straw which was buried in

four arable soils, an allotment soil, and a compost, was investigated by Sadasivan (105). He found that *Fusarium culmorum* and *Mucor* spp. were dominant in the earlier stages of straw colonization, but that these organisms eventually were replaced by *Penicillium* spp. The development of the *Penicillium* spp. was favored by previously autoclaving the straw in 2 per cent sodium nitrate solution.

Successions involving 230 fungus species were observed by Mangelot (106) on the surface and interior of standing and fallen trees in various stages of decay and also on beech sawdust.

Recently the ecological nature of sequential relationships among societies of fungi pathogenic on stolons of white clover was investigated by Garren (108) in Alabama. He noted that 50 per cent or more of the isolates from injured stolon tissues were *Fusarium* spp. and *Rhizoctonia solani*; less frequently isolated were *Sclerotium baticola*, *Sclerotium rolfii*, *Alternaria tenuis*, *Curvularia trifolii*, *Colletotrichum graminicola*, and *Pythium* spp. The seasonal succession of these fungi on white clover stolons, in terms of defoliation caused by stolon injury, was characterized by a maximum for *Rhizoctonia* in late August, and for *Fusarium* spp. in late September. *Curvularia* accounted for the major damage in October whereas *C. graminicola* reached a maximum in early November. Stolon damage by *Pythium* accounted for most of the total defoliation in late December.

Garren (107) also studied the seasonal successions of fungi pathogenic on leaves of 40 different clonal lines of white clover. He determined in isolation cultures, made at monthly intervals, the per cent of the total isolations of each fungus. Fungi isolated from diseased leaf tissue during the first 12 months were *Alternaria tenuis*, *Sclerotinia trifoliorum*, *Pseudopeziza trifolii*, *Curvularia trifolii*, *Stagnospora meliloti*, *Colletotrichum graminicola*, and *Colletotrichum destructivum*. During February, approximately 81 per cent of the isolates were *A. tenuis* while 15 per cent were *C. destructivum*. However, in June, about 85 per cent were *C. destructivum* and 10 per cent were *A. tenuis*. In August, *C. graminicola* accounted for 50 per cent of the isolates whereas *C. destructivum* decreased to about 10 per cent and *A. tenuis* to 5 per cent.

Frequently, metabiotic phenomena have been observed on stored grain. Bottomley *et al.* (109) determined the changes in mold flora in corn stored 12 days at four moisture contents and under continuous aeration. After two days at seed moisture contents of 18.1 to 19.9 per cent, *Penicillium* sp. comprised 90 to 100 per cent of the total mold species isolated from the corn whereas in later isolations the concentrations of *Aspergillus glaucus*, *A. candidus*, *A. flavus*, and *A. tamarii* increased, with a corresponding decrease in *Penicillium* spp. Similarly, in isolations from corn stored at 23.4 to 24.7 per cent moisture content, *Penicillium* spp. constituted about 80 to 95 per cent of the isolates, but in later isolations the frequency of *Penicillium* decreased to 5 to 20 per cent. In contrast, *Aspergillus glaucus* increased from 0 to 100 per cent of the isolates in four days, then gradually decreased in later isolations. At moisture contents of 25.4 to 30.4 per cent and 30.2 to 42.5 per cent, *Asper-*

gillus spp. predominated whereas *Penicillium* sp. was initially high in frequency and then gradually decreased.

The influence of storage conditions upon the development of injurious molds on barley was determined by Tuite & Christensen (110). During storage periods up to 18 months at seed-moisture contents of 13.8 to 14.2 per cent, *Aspergillus restrictus* developed slowly and invaded the germs, whereas at moisture contents of 15 to 17 per cent, *Aspergillus repens*, *A. amstelodami*, and *A. ruber* became the dominant flora. Other fungi, such as *Alternaria*, *Cladosporium* and *Fusarium*, which were originally present in the seed did not persist at the higher moisture contents.

Some of the most interesting and orderly successions of fungi have been found in apple juice and various pickle brines. The relative incidence and succession of spoilage molds in apple juice at different stages of processing, were determined by Marshall & Walkley (111). They found that for apples in "silo stage," *Penicillium expansum* was present in 35.6 per cent of the platings, *Aspergillus fumigatus* in 6.2 per cent, and *Aspergillus niger* in 6.0 per cent. For the "juice fresh from press stage," *P. expansum* occurred in 30.0 per cent of the platings, *A. niger* in 7.5 per cent, *Penicillium cyclopium* in 6.0 per cent, and *A. fumigatus* in 5.5 per cent. After the juice was in storage tanks for 18 months, *A. fumigatus* had increased and was found in 26.0 per cent whereas *P. expansum* had decreased and was found in 20.2 per cent of the platings. In this same processing stage *Cladosporium herbarum* and *A. niger* increased to 25.0 and 10.1 per cent, respectively. In the final processing stage "pasteurized bottled juice," *A. fumigatus* and *P. expansum* decreased to 17.2 per cent and 19.7 per cent, respectively, whereas *Paecilomyces varioti* and *A. niger* increased to 5.8 and 11.8 per cent, respectively. In addition to the fungi mentioned, the relative incidence of 29 other molds was determined. Marshall & Walkley (111) also found that the incidence of yeasts relative to the molds progressively increased throughout the processing stages whereas the frequency of bacteria diminished.

The changes in kinds and amounts of different yeasts in aging commercial cucumber brines were determined by Etchells *et al.* (112, 113). They noted that during the first 28 days of aging, *Torulopsis caroliniana* predominated, reaching a peak concentration the sixteenth day of approximately 1 million yeasts per ml. of brine. Living cells of *T. caroliniana* persisted in the brines for about 43 days. About the sixth day, *Hansenula subpelliculosa* began to increase, reaching a maximum concentration of 36,000 yeasts per ml. of brine the twenty-second day. After the fifty-seventh day, living cells of this yeast were not found. Another yeast, *Zygosaccharomyces globiformis*, which appeared the twenty-fourth day, increased to a maximum of 31,000 yeasts per ml. of brine and then decreased. After the sixty-seventh day living cells of this yeast were undetectable in the brines. The most persistent yeast was *Brettanomyces versatilis* which appeared about the twenty-first day reaching a peak concentration of 33,000 yeasts per ml. of brine the thirty-second day. This yeast then persisted in the brines for over three months.

The above examples of metabiosis have been limited to fungal successions; however, microbial successions involving bacteria in substances such as milk are also well known. The elusive nature of the changes which constantly occur in various substrates and cause these modified substrates to continually select and inhibit different organisms are largely unknown. Nevertheless, the accumulation of various metabolic products and staling substances, the moisture content (109, 110) and temperature (107, 108), the concentration of oxygen and carbon dioxide (111), as well as the nutritional and spatial limitations of the substrate (99), probably all contribute more or less to the reshuffling of substrate properties which are most favorable to a select few of the waiting organisms.

Synergism.—Fungal relationships in which the combined activities of two or more participating fungi cause an effect which is greater than that produced by any of the fungi alone are generally termed synergistic. Such relationships have been reflected by disease development in plants, by the amount and kinds of growth and sporulation of fungi on different substrates, and by the synthesis of various metabolites. However, synergistic phenomena in microbial associations are not restricted to the above categories and may be represented by any response which is detectable.

The importance of investigations on the effects of known mixtures of microorganisms has been stressed by Fawcett (114) who demonstrated that inoculations of certain mixtures of fungus pathogens into citrus bark caused greater injury than inoculations with any single organism in a mixture (115). Thus, injury caused by inoculations with mixtures of *Diplodia natalensis* and *Colletotrichum gloeosporioides* was more severe than that caused by inoculations of either pathogen singly.

Moreover (116), when bits of pure cultures of *Pythiacystis citrophthora*, *Phytophthora citrophthora*, and *Fusarium* sp. were placed side by side in cuts in bark of lemon trees, the severity of the resulting gummosis disease was greater than that caused by inoculations of the pathogens alone.

In further studies Savastano & Fawcett (2) determined the influence of inoculum concentration, constant and fluctuating temperatures, humidity, period elapsed between the time of picking and inoculation, fruit color, and resistance of the rind to puncture, on decay of lemons and oranges by single or mixed inoculation of citrus pathogens. Mixed inoculum consisting of *Penicillium italicum*, *P. digitatum*, and *A. niger* produced higher rates of decay than inoculations of any of the fungi alone. In addition, *Oospora citri-aurantii* in mixed cultures with *P. italicum* and *P. digitatum* caused a higher rate of decay, especially on lemons, than inoculations of any of the fungi alone.

Gemmel (117) also found that citrus fruits rotted more rapidly through the joint action of *P. digitatum* and *O. citri-aurantii* than through the action of either alone. He found in cultural studies that *P. digitatum* produced a thermostable nitrogenous substance that stimulated the growth of *O. citri-aurantii*. He also found that in mixed cultures, the two fungi had a much

longer growth period, and he concluded that the greater rot in citrus fruits brought about by the interaction of these fungi had two causes: stimulation of the *Oospora* by a by-product of the *Penicillium*, and the removal of a staling substance which would have hindered the growth of the *Penicillium*.

The influence of different substrates on the virulence of two pathogens of citrus was studied by Osteraas (3). She observed that inoculations of lemons or limes with either *P. digitatum* or an unidentified nonsporulating fungus resulted in considerable fruit deterioration, whereas inoculations with both fungi together caused much less rot. However, inoculations of navel oranges with both fungi together resulted in greater fruit deterioration than from inoculations with either of the pathogens alone. Similar results were found by Savastano & Fawcett (2) with various combinations of pathogens on lemons and oranges.

In other studies on synergistic effects, Geach (118) found that the combined action of *Urocystis tritici* and *Fusarium culmorum* caused damping-off in 37.8 per cent of wheat seedlings, whereas *Urocystis* alone caused 3.6 per cent and *Fusarium* 1.8 per cent. This type of synergism was also noted by Hawn & Cormack (119), who found that the severity of crown bud rot in alfalfa was significantly increased when inoculum of *Rhizoctonia solani* was mixed with that of *Fusarium acuminatum* and *F. avenaceum*. Michaelson (120) noted that corn smut predisposed corn to stalk rot caused by *Giberella zeae* and *Diplodia zeae*.

A synergistic response was also observed by Weir (121) when cankers formed by *Plowrightia morbosa* on *Prunus* and *Amelanchier* developed large burls following infection by *Fomes* sp.

In studies of physiologic specialization in loose smut of wheat and barley, Cherewick (122) found that the association of ergot conidia with loose smut spores that are naturally disseminated by air currents not only provides one method of ergot dissemination, but may have some relationship to the high prevalence of ergot in loose smut susceptible varieties such as Montcalm barley and Lee wheat.

Increased growth and sporulation of various fungi in mixed cultures have been frequently noted; however, in regard to this type of synergistic response, the studies of Lillian E. Hawker and F. D. Heald and their co-workers are particularly noteworthy. Raper (94) and Lilly & Barnett (123) have adequately reviewed the early literature on these effects.

Chemical control of conjugation in three *Zygosaccharomyces* spp. which conjugate only sparingly in pure culture, was studied by Nickerson & Thimann (124) who found that these yeasts sporulated more frequently in dual cultures with *A. niger*. Increased conjugation in mixed cultures was caused by an unidentified substance excreted into the medium by *A. niger*. In later studies (125), the active principle was shown to consist of at least two fractions, one having the properties of an organic acid and the other the properties of a vitamin in the B-complex. The conjugation-promoting action of the substances from the *A. niger* cultures was duplicated by a synthetic

mixture of glutaric acid and riboflavin; however, it was not definitely established whether these compounds were the same as the unidentified substances from *A. niger*.

Niemann (126) noted that *Trichoderma viride* greatly stimulated the germination of chlamydospores of *Tilletia caries* when both species were planted together on horse dung decoction agar. Similar responses were observed by Wood (127) who found that increased sporulation of *Botrytis cinerea* frequently occurred when it was grown in dual cultures with *T. viride*, *Penicillium* spp. or *Fusarium* spp. Growth of *Sclerotium rolfii* also increased when it was grown in the presence of other soil microorganisms on a thiamine-deficient medium (128).

Of particular interest is the recent study by Nickerson & Chung (129) who found evidence that the filamentous growth of a morphological mutant of *Candida albicans*, under conditions that permit the parent strain to grow as a yeast, is caused by a genetic block in its mechanism of cellular division. This block apparently is caused by a breakdown in an intracellular sulphhydryl-maintenance reaction. A partial restoration of the division mechanism in the mutant strain was obtained by the addition of cysteine or of material diffusing from streak cultures of the parent strain of *C. albicans*.

Synergistic interactions between morphogenetically deficient variants of the slime mold *Dictyostelium discoideum* were observed by Sussman (130). He found that in a mixed population of two deficient variants (induced with ultraviolet radiation), each achieved a level of morphogenesis significantly higher than either partner could accomplish alone. In some mixtures, mature fruiting structures with viable spores were produced. The nature and intensity of the synergistic response was dependent on the proportions of cells of the deficient variants in a mixed population. Thus, when a suspension of washed myxamoebae which contained a ratio of 10 plasmodia of variant Fr-1, to 1 of Agg-204 were plated on minimal agar, no fruiting occurred and only fruitless aggregates developed. Yet, when an equal number of myxamoebae of each variant or when an excess of Agg-204 plasmodia were present extensive fruiting occurred. Similarly when a mixture of Fr-1 and Agg-53 Myxamoebae contained a high ratio of Fr-1 plasmodia, only flat migrating aggregates were formed. In contrast, when an equal number of plasmodia of each variant or when an excess of Agg-53 were present, mature fruits were formed. In subsequent studies, an analysis of spores from synergistic fruitings and resulting clones was made to determine if syngamy had occurred before the aggregation of myxamoebae. No recombinant types were found among the spores; however, Sussman (130) discovered that a partial synergistic response resulted with a dialysis membrane separating deficient variants, but in no case did mature fruiting structures develop under these conditions. He concluded that in these instances syngamy was either very rare or non-existent and that at least part of the synergistic response resulted from an exchange of metabolic products.

In other studies, Bitancourt & Rossetti (131) found that *Mucor spinosus*

produced a gas which greatly stimulated the growth of *Phytophthora citrophthora* in dual cultures on potato-dextrose agar. In addition, the growth response of *Mucor* was also evident when separate cultures of these fungi shared the same gaseous environment.

A unique association of the fungi *Mortierella alpina* and two unidentified imperfect species all isolated from the roots of yellow birch (*Betula lutea*) was discovered by Redmond & Cutter (132). They found that the two unidentified fungi, when cultured together, synthesized a heat stable product which strongly inhibited the growth of *M. alpina*. When grown alone neither of the unknown species produced detectable amounts of this substance. The notion that useful antibiotic substances might be obtained through other synergistic associations was suggested.

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NUTRITION OF BACTERIA AND FUNGI^{1,2}

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A previous review by Cheldelin & King (45) concluded that the rich "mother lode" of new growth factors is approaching exhaustion. The statement is an interesting one for speculation [Wright (257)] but it must be admitted that no outstanding "nuggets" were unearthed in 1955. Instead, activity centered, for the most part, around exploitation of previous gains by less sensational achievements along other lines. Broad fields receiving special attention include: (a) nutritional investigations of previously neglected, obscure species, (b) nutritional requirements for processes other than growth, (c) biogenesis of growth factors, (d) role of known growth factors in enzymatic reactions, (e) production of growth factor requirements by mutation, (f) antimetabolite relationships, and (g) studies on the occurrence and function of derived forms of previously characterized growth factors.

THIAMINE

A new microbiological method for the determination of thiamine based on the essential nature of the vitamin for *Kloeckera brevis* has been proposed by Hoff-Jørgensen & Hansen (96). The method is claimed to be more satisfactory than a previous one employing *Lactobacillus fermentum*. The moieties of thiamine are without microbiological activity in the new assay.

Evidence has been presented by Harris (89) for the existence of two pathways of thiamine biosynthesis in *Neurospora crassa*. One pathway involves the direct condensation of the pyrimidine and thiazole moieties. The second and predominant pathway appears to involve the condensation of the pyrimidine moiety with a thiazole precursor followed by conversion of the intermediate into thiamine.

VITAMIN B₆

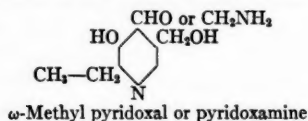
Vitamin B₆ (as pyridoxal phosphate) was shown by Snell, and Gunsalus & Wood (101, 214, 250) to be a coenzyme for the synthesis of D-alanine. In the absence of this enzyme D-alanine is required as a growth factor for a number of lactobacilli. Snell *et al.* (215) have now shown that this amino acid exists in lactic acid bacteria in two forms. One form is extractable with hot trichloroacetic acid where it exists in the form of one or more unidentified derivatives. The other form is firmly bound in the insoluble portion of

¹ The survey of literature pertaining to this review was completed in December 1955.

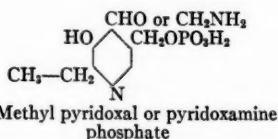
² The following abbreviations are used: DNA (deoxyribonucleic acid); PAB (*p*-aminobenzoic acid); POB (*p*-hydroxybenzoic acid); PPA (prephenic acid); CoC (coenzyme C).

the cell from which it is released by acid hydrolysis at essentially the same rate as ninhydrin reactive material. D- α -Amino-n-butyric acid replaces D-alanine as a growth factor for a number of lactobacilli. Bacteria grown on D- α -amino-n-butyric acid contain this amino acid instead of D-alanine, indicating that the analog substitutes for D-alanine instead of being converted to it.

Olivard & Snell (161, 162) have studied the activity of a number of ω -methyl analogs of vitamin B₆ on the growth of *Streptococcus fecalis* under conditions where the synthesis of D-alanine or the L-isomers of alanine, leucine, isoleucine, valine, phenylalanine, tyrosine, methionine, serine, or cysteine is the rate limiting reaction.



ω -Methyl pyridoxal or pyridoxamine



ω -Methyl pyridoxal or pyridoxamine phosphate

These compounds are active but less so than the corresponding natural isomers in all the reactions studied except L-cysteine and L-alanine synthesis. Here the compounds are antimetabolites. *In vitro* enzymatic systems were also studied. The use of a vitamin B₆ mutant of *Escherichia coli* in the assay of this group of vitamins has been studied by Diding (54). Denman *et al.* (52) have established in studies with a pyridoxine deficient mutant of *E. coli* that pyridoxal phosphate is a coenzyme for diaminopimelic acid decarboxylase. The function of this enzyme is to supply the cell with lysine.

VITAMIN B₁₂

Bardos *et al.* (22) have observed that the vitamin B₁₂ (or thymidine) requirement of *Lactobacillus leichmannii* is, at least between certain limits of concentration, a direct function of the ionic strength of the medium. As a possible explanation for the high requirement for vitamin B₁₂ in the presence of salt it was postulated that a vitamin B₁₂ "apoenzyme" combination exists that may be "salted out" of solution to yield enzymatically inactive material. Most "simple" proteins require a greater salt concentration than used in these experiments, but deoxyribonucleoprotein from sea-urchin eggs has an ionic strength-solubility curve resembling that calculated for a hypothetical vitamin B₁₂ deoxyribonucleoprotein "apoenzyme" complex. The data are interpreted as indicating the possible existence of such a complex which acts as an autocatalyst in the biosynthesis of new deoxyribonucleoprotein.

A number of reports (some of them in abstract form only) have appeared on the microbiological determination of vitamin B₁₂. A collaborative report of the American Association of Agricultural Chemists recommends that a method slightly modified from that of the U.S. Pharmacopeia XIV, which employs *L. leichmannii* as the assay organism, be made official for materials containing 0.1 μ g. or more of vitamin B₁₂ per gm. or ml. It is further recom-

mended that methods for materials containing less than the specified amount of vitamin B₁₂, or materials containing microbiologically inactive forms of vitamin B₁₂ receive additional study [Krieger (124)]. Abstracts of a meeting devoted to the biological and microbiological methods for the determination of vitamin B₁₂ held by the Society for Analytical Chemistry (British) are available (16), and the papers have been summarized briefly (17). Specific papers concerned with the microbiological determinations of vitamin B₁₂ in serum [Boger *et al.* (30)], serum and urine [Killander (119)], bovine rumen liquor and feces [Moinuddin & Bentley (151)], and milk (18) have appeared. Additional specific details of the *Euglena gracilis* (125), *Ochromonas malhamensis* (64, 108, 147) and *E. coli* (133) methods have been published.

PANTOTHENIC ACID AND PANTETHEINE

The extent to which a series of pantothenic acid peptides promote the growth of pantothenate or pantetheine-requiring bacteria, or stimulate the synthesis of coenzyme A in *Lactobacillus arabinosus* has been studied by Pierpoint *et al.* (167). Only pantothenyl glycine is an effective substitute for pantothenic acid in the growth of *L. arabinosus*. None of the compounds substituted for pantetheine in the growth of *Lactobacillus helveticus*.

The synthesis and microbiological evaluation of a number of new derivatives of pantothenic acid, pantothenamide, pantothenyl glycine, pantothenylleucine, and pantothenylhistidine have been described by Brown *et al.* (39). Utilization by bacteria was not such as to indicate any metabolic significance for the compounds. None was an antimetabolite of pantothenic acid or pantetheine.

Eight new derivatives of β -aletheine (β -aletheine is N-(β -alanyl)-2-aminoethyl mercaptan and β -alethine is the disulfide form) and pantetheine have been described by Stewart *et al.* (219). Although none was as active as pantothenic acid or pantetheine for an appropriate microorganism, interesting instances of growth promotion were observed. Oxypantetheine (N-pantothenylethanolamine) was found to be an active antimetabolite of pantetheine.

A kinase from *Proteus morganii* that phosphorylates pantothenic acid, pantetheine, and pantethine with equal ease has been studied in detail by Ward *et al.* (240). The enzyme resembles in many respects a previously known pantetheine kinase from pigeon liver, but there are also points of dissimilarity.

The phosphorylation of pantothenic acid by an enzyme of *L. arabinosus* has been investigated by Pierpoint *et al.* (168). The product of the reaction, pantothenic acid 4'-phosphate, is unavailable as a source of pantothenate for bacteria [King & Strong (120)]. Pantothenic acid may be recovered following phosphatase treatment.

Ginoza & Altenbern (76) have shown that *Brucella abortus* (strain 19) possesses a pantoate- β -alanine coupling enzyme and have studied the en-

zyme in detail. Since *B. abortus* ordinarily is considered to require pantothenate for good growth, or at least to be greatly stimulated by the vitamin, it is concluded that the enzyme is not fully functional in the cell.

FOLIC AND FOLINIC ACIDS

A new assay for folic acid based on the essential nature of this vitamin for a strain of *Bacillus coagulans* has been proposed by Baker *et al.* (20). Advantages of this method over pre-existing ones are: (a) the organism responds to conjugates of folic acid so that pretreatment of at least some materials with conjugase is obviated, (b) the organism is a thermophile so that aseptic techniques are not required to prevent ordinary contamination, and (c) a simple, completely synthetic, basal medium is all that is required for adequate growth. Disadvantages of the method are: (a) the organism responds to p-aminobenzoic acid (but not its glycine conjugate p-aminohippuric acid), but this response may be circumvented by the addition of sulfanilamide to the basal medium, and (b) extraneous components of natural material might be expected to show some nonspecific stimulation with the simplified medium recommended.

A number of new naturally-occurring derivatives of folic acid or folinic acid (citrovorum factor) with significance in bacterial nutrition or metabolism have recently been discovered. Hakala & Welch (85) have observed the existence of a compound in *Bacillus subtilis* cells which, on the basis of its microbiological activity both before and after treatment with chick pancreas and hog kidney conjugase and its behavior on paper chromatography, is believed to be 5-formyl-5,6,7,8-tetrahydropteroyl- γ -glutamyl- γ -glutamyl-glutamic acid. Similarly Zakrzewski & Nichol (266) have found that intact cells or extracts of *S. fecalis* convert folinic acid into a compound also considered to be 5-formyl-5,6,7,8-tetrahydropteroyl- γ -glutamyl- γ -glutamyl-glutamic acid (folinic acid triglutamate). More extended studies by the same group [Nichol *et al.* (159)] have shown the enzymatic formation by *S. fecalis* cells of a heat labile derivative of folinic acid which is converted into the stable folinic acid by heat. The compound is considered closer to the "coenzyme" form of folic acid than is folinic acid. That the heat labile derivative is N¹⁰-formyltetrahydrofolic acid may be inferred from this paper. The paper contains, in addition, an excellent extended discussion of the present status of naturally occurring derivatives of folic and folinic acids.

A new cofactor termed CoC required for the conversion of serine to glycine has been described by B. Wright (254, 255). The factor is active at catalytic levels while folinic acid and tetrahydrofolic acid are active at substrate levels. Folic acid and anhydroleucovorin are inactive. Diphosphopyridine nucleotide is required for CoC activity. The activity of this factor in satisfying the folic or folinic acid requirements of bacteria was not specified. Subsequent study by B. Wright (256) indicates that CoC preparations from *Clostridium cylindrosporum* may be separated into a number of compounds which are polyglutamyl derivatives containing additional amino acids including glycine, serine, alanine, and a new unknown amino acid.

A compound that appears to be closely related to CoC at least in function has been isolated from enzymatic reaction mixtures and identified as N¹⁰-formyltetrahydrofolic acid by Jaenicke (105) and Greenberg *et al.* (80). This compound (a) transformylates to imidazolecarboxamide ribotide in the absence of adenosinetriphosphate, (b) is converted to the N⁵-N¹⁰-imidazolinium derivative of formyltetrahydrofolic acid, (c) is oxidized to a compound having the properties of N¹⁰-formyldihydrofolic acid, and (d) is converted chemically to folinic acid. Whether or not N¹⁰-formyltetrahydrofolic acid is the natural carrier of formate is unknown, but the above studies strengthen the concept first proposed by Gordon *et al.* (79) that N¹⁰-derivatives are the active cofactor.

Usdin & Toennies (230) have continued their studies on the folic acid-active substances of blood hemolysates and have shown that six or eight of such factors exist. Considerable purification of the active mixture was achieved, but it was not possible to relate more than one or two of the components to known derivatives of folic acid by paper chromatographic methods.

The isolation of 5-formyl-5,6,7,8-tetrahydrofolic acid in which the formyl carbon is labelled after incubating an A-methopterin resistant strain of *S. fecalis* with folic acid and C¹⁴ formate has been described by Zakrzewski & Nichol (267). The compound has twice the activity of synthetic folinic acid.

The inactivation of folic acid antimetabolites by microorganisms with no requirement for folic acid has been studied by Webb (243). Recovery from inhibition was accompanied by oxidative cleavage of the compounds to yield pteridine-6-carboxylic acid, 6-hydroxymethylpteridine or both, and p-aminobenzoylglutamic acid. Neither free p-aminobenzoic acid nor glutamic acid was detected, so that hydrolysis is apparently specific for the C⁹ to N¹⁰ linkage.

BIOTIN

Micrococcus sodonensis was proposed as an assay organism for biotin by Aaronson (1). Its requirement for biotin or similar compounds is very low (ca 1×10^{-6} μ g. per ml.) and its other requirements are all met with pure compounds. Evidence for the conclusion that biocytin is more active than biotin for this organism was not convincing and could have been explained by some slight oxidation of the biotin used to microbiologically unavailable sulfoxides or sulfone, or by only slight errors in weighing or diluting the active compounds.

Schaefer *et al.* (196) have described strains of *Mycobacterium tuberculosis* with an absolute requirement for biotin on an oleic acid-albumin agar medium. Incubation under increased CO₂ tension, which has been known for some time to stimulate growth of the tubercle bacillus, abolished the biotin requirement. The biotin analog 4-(imidazolidone-2) caproic acid inhibited growth of these strains. All levels of inhibition were reversed with biotin while increased CO₂ tension reversed smaller amounts. The presence of oleic acid in the medium increased the requirement for biotin or carbon

dioxide. This finding is opposed to that observed with certain lactobacilli where the biotin requirement is sometimes abolished with oleic acid and CO_2 .

The biotin requirement of some members of the genus *Propionibacterium* may be satisfied with either oxybiotin or desthiobiotin according to Lichstein (126). For one species desthiobiotin is more active than biotin. A pronounced lag occurs in the utilization of oxybiotin suggesting some alteration in structure prior to utilization. With one strain it was observed that biotin which had been stored in the refrigerator or autoclaved with the basal medium was markedly less active than fresh material added aseptically after autoclaving. Possibly this decrease in activity for one strain is the result of partial oxidation of biotin to a mixture of the two diastereoisomeric sulfoxides. The d-sulfoxide which is produced in the largest amount by *in vitro* oxidation is, for example, as active as biotin for *L. arabinosus* and *Saccharomyces cerevisiae* but is essentially inactive for *Lactobacillus casei* [Melville *et al.* (144)]. Considerations of this sort should be made in any quantitative studies with biotin and derivatives.

A number of compounds have been examined by Wright *et al.* (259) for activity as precursors of biotin, biotin 1-sulfoxide or both, in biosynthetic studies with *Aspergillus niger*. The odd carbon straight chain dicarboxylic acids pimelic and azelaic are precursors. The even chain acids suberic and sebacic as well as acids shorter than pimelic are inactive. A number of miscellaneous compounds with some relationship to pimelic acid were also inactive. Cystine did not augment the dicarboxylic acid effect thus indicating that cystine is probably not a structural precursor of biotin as might be predicted from structural considerations.

Factors influencing biotin sulfone inhibition of *L. arabinosus* have been studied by Ravel & Shive (184). Compounds exerting a reversing effect on sulfone inhibition include aspartic acid, oleic acid, threonine, lysine, uracil, and an unknown metabolite. It is suggested that biotin functions in the biosynthesis of the above compounds or that these compounds are involved in the utilization of biotin.

Lichstein & Boyd (128) have presented evidence that in *S. cerevisiae* biotin functions in two ways: (a) in the synthesis of an enzyme(s), and (b) in the dissimilation of carbohydrate. Since ultraviolet light-treated cells were stimulated in glucose oxidation by biotin but not by aspartic acid, while untreated cells were stimulated by biotin or aspartic acid it was concluded that the two kinds of effects produced by biotin on the rate of glucose oxidation had been separated. While the reviewer concedes that biotin may have more than one function in metabolism this paper falls short of proving it.

The presence of bound biotin in purified preparations of oxaloacetate carboxylase has been claimed by Lichstein (127) on the basis of microbiological studies primarily with *S. cerevisiae* (strain 139) as the assay organism. This work may be criticized for a number of reasons, and the conclusion is not generally accepted by those conversant in the field. A new lead to the mechanism of action of biotin follows from a note by Katsuki (112). Biotin deficient cultures of *Piricularia oryzae* were found to accumulate considerable amounts of dimethylpyruvic acid.

LIPIC ACID

Fuller *et al.* (72) have observed that *Chlorella* fed lipoic acid converts the compound under aerobic conditions into a biologically active, lipid soluble (high R_f) derivative. The compound can be converted back to lipoic acid with acid. The derivative is a major metabolic derivative of lipoic acid in the cell and may have some significance in photosynthesis since lipoic acid must be metabolized aerobically by *Scenedesmus obliquus* before any stimulation of the Hill reaction is observed. Calvin is reported to have stated in a symposium meeting (198) that in this compound, the carboxyl group of lipoic acid is bound in an ester linkage to a hydroxyl group of glycerol.

The possible relationship of lipoic acid to photosynthesis (outside the scope of this review) has been discussed by Bradley & Calvin (34).

AMINO ACID BIOSYNTHESIS AND NUTRITION

Methionine.—The activity of a number of compounds related to methionine in promoting growth of auxotrophic mutants of *E. coli* has been studied by Kalan & Ceithaml (109). Similar studies with *Streptococcus lactis* have been reported by Kizer *et al.* (122). The results of both groups point to the existence of a pathway of biogenesis in bacteria similar to that of the rat involving a general sequence of: homoserine \rightarrow cysteine \rightarrow cystathionine \rightarrow 3 carbon fragment \rightarrow homocysteine \rightarrow methionine.

A strain of *E. coli* was found by Bird & Gots (29) that requires either methionine or *p*-amino-benzoic acid (PAB) at 37°C. but not at 20–25°C. The requirement was considered the result of a deleterious reaction at the higher temperature that interferes with the function of PAB in the biosynthesis of methionine.

Growth and biosynthetic studies of Shapiro (199) with methionine auxotrophs of *Aerobacter aerogenes* indicate methionine synthesis from homocysteine plus methylmethionine sulfonium salt but essentially no synthesis from either component alone. Similarly, Schlenk & DePalma (197) have shown that in the growth of *Torulopsis utilis* methylmethionine sulfonium salt is inferior to methionine as a precursor of methylthioadenine. Homocysteine is inactive. However, in the presence of the two compounds methylmethionine sulfonium salt and homocysteine, methylthioadenine synthesis is greater than can be obtained with any level of methionine. Methylmethionine sulfonium salt had been shown previously to be superior to methionine in promoting growth of a number of microbial species [McRorie *et al.* (135)].

Some properties of a compound that accumulates during growth of a "leaky" methionine-requiring mutant of *N. crassa* are recorded by Carlson & McRorie (43). The compound was shown to be distinct from known intermediates in methionine biosynthesis. The biological activity of the compound for a series of other mutants of *N. crassa* indicates the following sequence:

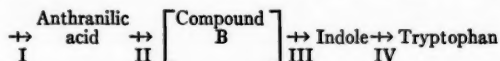
Homocysteine → New intermediate → Methionine

γ -Methio- α -hydroxybutyric acid is as active as methionine in satisfying the sulfur amino acid requirement of *L. arabinosus* according to Shiota & Clark (200). L-Cysteine, L-cystathionine, DL-homocysteine, and DL-homocystine were also active in that order. Serine was required for growth when the sulfur source was methionine or γ -methio- α -hydroxy-n-butyric acid but it could be omitted from the medium in the presence of cysteine or cystathionine. *p*-Aminobenzoic acid (PAB) was required in the presence of cysteine, homocysteine, or cystathionine but not in the presence of methionine or its α -hydroxy analog. This information suggests that it may be possible to "stabilize" the requirement of *L. arabinosus* for PAB by using a methionine-free medium. This procedure might permit reliable assays for PAB to be obtained. If so, one of the most troublesome and controversial problems in microbiological assays of a decade ago would have been resolved.

Reddi (186) has shown that when *Pseudomonas hydrophila* is grown in a synthetic medium with added methionine more dry weight of cells, more cell protein, more RNA and more incorporation of P^{32} into RNA nucleotides was obtained. DNA synthesis was not influenced. This is another study where a positive correlation between protein synthesis and RNA synthesis indicates that the two processes are interrelated.

Tryptophan.—The synthesis of indole and anthranilic acid by resting cells of a number of *E. coli* mutants blocked at various stages in the biosynthesis of tryptophan has been studied by Gibson *et al.* (73). A mutant that grew with tryptophan or indole accumulates an unidentified compound with absorption maxima at 278 and 287 μ . The complete absorption spectrum is slightly different from that of indole. Serine stimulates the formation of this compound, and it was concluded that serine has a function in tryptophan synthesis in addition to its role as a precursor of the "alanine" moiety.

A study of tryptophan auxotrophs of *Salmonella typhimurium* by Brenner (35) has indicated that these mutants may be classified into four groups (I–IV) genetically blocked in the biosynthesis of tryptophan as follows:



The nature of compound B is unknown but it has no growth-promoting activity for mutants blocked in earlier stages and may, in fact, be produced by a side reaction from the normal intermediate. Compound B has an ultraviolet absorption maximum at 278 μ . and may be identical with the unknown intermediate of Gibson *et al.* (73).

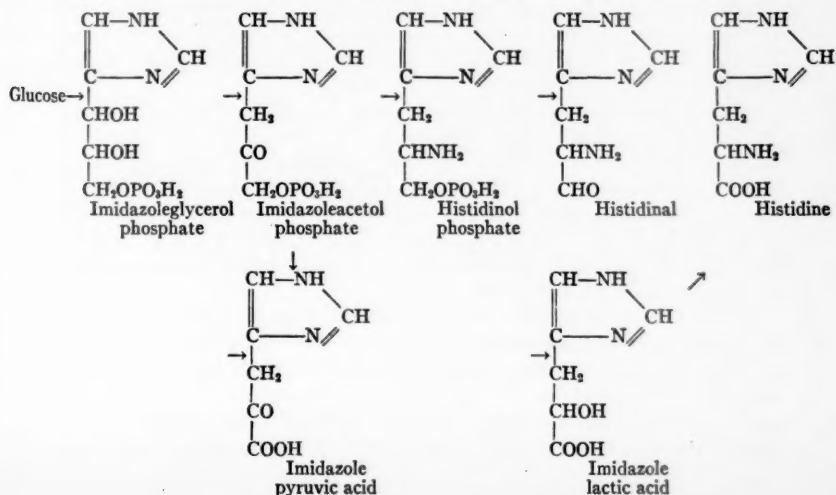
Phenylalanine and tyrosine.—Phenylalanine and tyrosine are required by *L. arabinosus* in the absence of CO_2 but are synthesized in a CO_2 atmosphere (Lyman *et al.* (132)). Ory & Lyman (163) have now shown that in the presence of CO_2 these amino acids are synthesized with either glucose, mannose, or fructose as the carbohydrate component of the medium. Other carbohydrates and related derivatives are inactive. Shikimic acid can substitute for

the carbohydrate and for CO_2 . However, tyrosine isolated from cells grown on C^{13}O_2 and C^{14} -glucose contained no C^{14} although the C^{14} content of the tyrosine equaled that of the original glucose. Obviously if CO_2 were required in the synthesis of an intermediate it was lost at a later step in the synthetic pathway. Glycolysis intermediates supply the side chain of tyrosine and phenylalanine since amino acid synthesis is stimulated with these intermediates in the presence but not the absence of shikimic acid.

Histidine.—According to Westley & Ceithaml (246) two pathways for histidine biosynthesis in *E. coli* mutants are possible. The natural pathway involves L-histidinol while a pathway involving L-imidazolelactic acid is also possible. Distribution of radioactivity in histidinol accumulated by an *E. coli* mutant grown on glucose-1- C^{14} or -6- C^{14} was such as to indicate the condensation of a 2-carbon and a 3-carbon fragment arising from the glycolytic cycle. Similarly in histidineless mutants of *S. typhimurium* the primary pathway of histidine synthesis involves the phosphate ester of imidazole glycerol, imidazole acetol, and histidinol according to Hartman (90). A shunt of secondary importance originating in imidazole acetol involves imidazole lactic and pyruvic acids (see following reactions). Enzymatic studies of Adams (5) with preparations from *E. coli*, yeast, and *Arthrobacter* indicate that L-histidinol is an intermediate in the oxidation of histidinol to histidine.

Previous studies on histidine biosynthesis in *N. crassa* have been extended by Ames & Mitchell (14) who have isolated and characterized the following intermediates from appropriate mutants: D-erythro-imidazoleglycerol phosphate, imidazoleacetol phosphate, and L-histidinol phosphate.

The biosynthesis of histidine in bacteria and fungi (subject to certain exceptions noted subsequently) may be presumed to occur as summarized here.



An interesting histidineless mutant of *E. coli* that satisfies its histidine requirement with various purines has been described by Luzzati & Guthrie (131). A similar phenomenon was observed earlier in lactobacilli by Broquist & Snell (37). Mitoma & Snell (149) have now isolated histidine or histamine from lactobacilli grown in the presence of two, four or eight labelled C^{14} guanine. Carbon 2 of guanine is readily incorporated into the amidine carbon (position two) of the imidazole ring of histidine and is actually a more direct precursor than is formate. Incorporation of formate into this position requires folic acid but guanine-2 incorporation occurred in cells grown without folic acid and would not appear to proceed via free formate as an intermediate.

A histidineless mutant of *A. aerogenes* was found by Magasanik (137) to require 20 times as much histidine when growing on any one of a number of miscellaneous compounds such as *myoinositol* as a source of carbon as when growing on glucose. When grown on inositol adaptive enzymes were formed which destroyed histidine. Glucose was shown to be an inhibitor of this adaptive enzyme formation. Acyl derivatives of histidine did not induce the formation of adaptive enzymes and for this reason were more active than histidine in promoting growth of histidineless mutants grown on inositol. These findings may be of significance in explaining the enhanced activity of certain peptides over that of the component amino acids in promoting growth of certain amino acid-requiring bacteria. Some of the products of histidine degradation in *A. aerogenes* and *Pseudomonas fluorescens* are urocanic acid, formamidineoglutamate, glutamate and formamide or formyl glutamate and, finally, glutamate and formate [Magasanik & Bowser (138)].

Inhibition of histidine auxotrophs of *N. crassa* by various amino acid combinations has been studied by Mathieson & Catcheside (141). The inhibition observed was ascribed to an interference with histidine uptake. Accumulation of imidazole compounds was noted by one mutant but the compounds were not completely characterized.

Diaminopimelic acid.— α , ϵ -Diaminopimelic acid has been isolated by Ikawa & O'Barr (104) from *L. arabinosus* cells. No species other than the original *E. coli* mutant of Davis has been reported as yet that requires this amino acid. Hoare & Work (95) have shown that the meso and LL isomers of the amino acid are equally active in promoting growth of the above mutant. It was previously shown that diaminopimelic acid is inactive in satisfying the lysine requirements of *N. crassa* lysineless, *S. fecalis* and *Leuconostoc mesenteroides* [Wright & Cresson (258)].

A number of excellent reports and reviews have appeared on the biogenesis of the amino acids in microorganisms involving primarily isotopic-labelling experiments. These studies are slightly outside the scope of this review but because of their obvious relationship to amino acid nutrition, are summarized with authors and references: alanine [Wang *et al.* (239)]; Abelson & Vogel (4); arginine [Abelson & Vogel (4)]; cysteine [Abelson & Vogel

(4)]; glycine [Abelson & Vogel (4); Wang *et al.* (239)]; isoleucine [Strassman *et al.* (222); Adelberg (6, 7, 8); Umbarger & Brown (229); Abelson & Vogel (4); Weinhouse (244); Strassman & Weinhouse (223)]; leucine [Strassman *et al.* (221); Monsour & Wyss (152)]; lysine [Weinhouse (244); Abelson & Vogel (4)]; proline [Vogel (235); Abelson & Vogel (4); Yura & Vogel (265)]; serine [Abelson & Vogel (4); Meinhart & Simmonds (142); Wang *et al.* (239)]; threonine [Abelson & Vogel (4); Wang *et al.* (239); Wagner & Bergquist (236)]; valine [Strassman *et al.* (224); Weinhouse (244); Adelberg *et al.* (8, 9, 10); Wang *et al.* (239); Abelson & Vogel (4); Umbarger & Brown (229), Rafelson (179)].

AROMATIC BIOSYNTHESIS

Previous studies by Davis and collaborators (48) with nutritional mutants of *E. coli* have established the general pathway by which the aromatic rings of phenylalanine, tyrosine, tryptophan, *p*-aminobenzoic acid (PAB), and *p*-hydroxybenzoic acid (POB) originate. The sequence of known intermediates is: glucose→5-dehydroquinic acid (5DHQ)→5-dehydroshikimic acid (5DHS)→shikimic acid (SA)→prephenic acid (PPA)→aromatic compounds.

5-Dehydroquinic acid now appears to originate from a condensation of D-erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) to give 2-keto-3-deoxy-7-phospho-D-glucoheptonic acid as an intermediate [Srinivasan *et al.* (217); Sprinson (216); Kalan & Srinivasan (110)]. This pathway is compatible with previous studies indicating glucose as the ultimate precursor. Seduheptulose-1, 7-diphosphate, previously considered as an intermediate, is now considered to function only indirectly after conversion to the three and four carbon intermediates described above.

The conversion of 5-dehydroquinic acid to 5-dehydroshikimic acid involving the loss of one molecule of water is accomplished by a specific 5-dehydroquinase [Mitsuhashi & Davis (150)]. This enzyme has no demonstrable cofactor requirement. Shikimic acid originates from 5-dehydroshikimic acid through the action of the triphosphopyridine nucleotide-linked enzyme 5-dehydroshikimic acid reductase [Yaniv & Gilvarg (262)]. The unstable intermediate between shikimic acid and phenylalanine previously characterized as a new compound prephenic acid (PPA) (75, 245) is now considered to be a precursor only of phenylalanine and tyrosine. Another prior intermediate is postulated from which tryptophan, *p*-aminobenzoic acid (PAB) and *p*-hydroxybenzoic acid (POB) are derived [Davis (47)]. The present status of aromatic biosynthesis according to Davis (47, 49) is as follows:

PROTEINS AND PEPTIDES

Proteins.—The protein of mammalian sera required for the growth of certain pleuropneumonia-like organisms (212) has been identified by Smith *et al.* (211) as an α -1-lipoprotein containing only bound cholesterol and phospholipid. The factor is replaceable by the lipide-free protein, bovine serum

albumin, or β -lactoglobulin plus cholesteryl laurate and lecithin of animal origin.

Slade & Slamp (206) have been able to grow certain fastidious group A streptococci by the addition to a synthetic medium of crystalline ovalbumin which had been heated. A number of crude and crystalline proteins and peptides were inactive. The activity of the protein was considered as residing in "exposed" SH groups although other sulfhydryl compounds were inactive. The distribution of radioactivity between cells, media, etc. when C^{14} ovalbumin was used to promote growth was studied. The labelled protein was isolated from the oviduct of a hen fed $NaHC^{14}O_3$ (205). The protein was apparently degraded to smaller units prior to utilization.

A number of investigations have reported growth stimulation by proteins in a number of species including *B. subtilis* [Gjessing (77)].

Peptides.—The reversal by appropriate amino acid(s) or peptide(s) of inhibition by thienylalanine (phenylalanine antimetabolite), ethionine (methionine antimetabolite) or canavanine (arginine antimetabolite) has been studied in a number of bacteria by Kihara & Snell (118). In every instance where a source of amino acid was required for growth, a peptide was more active than the responsible amino acid as a reversing agent. Thienylalanine inhibition of wild type *E. coli* was reversed by much smaller amounts of phenylalanine than required for growth of a phenylalanineless mutant of this organism. Phenylalanine peptides were no more active than the free amino acid as reversing agents. It was concluded that in this instance synthesis rather than utilization of phenylalanine is inhibited by thienylalanine.

The utilization of a number of peptides of aspartic acid or asparagine, leucine, and glycine as a source of each amino acid has been studied in *L. mesenteroides* by Miller *et al.* (146). The peptides were utilized equally well or better than component amino acid or asparagine in most instances. The poor utilization of asparagine observed is attributed to inability of the free amide to reach the site of its metabolism in the cell.

Serylglycine was found by Meinhart & Simmonds (143) to be superior to serine, or serine plus glycine in promoting growth of a serineless mutant of *E. coli*. Similarly serylglycine was superior to other peptides or a mixture of glycine and serine in promoting growth of another mutant responding to serine or glycine.

The studies of Woolley and his group (145) on the nature of peptides stimulating growth of *L. casei* have continued with the isolation of an essentially pure peptide with high activity identified as serylhistidylleucylvalylglutamic acid (pentapeptide). The same group examined the activity of synthetic ocytoxin, highly purified vasopressin (252), and a number of synthetic peptides having some relationship to the sequence occurring in ocytoxin (253). Arguments were presented supporting the conclusions that activity is not associated with the presence in a peptide of any particular amino acid.

The growth-stimulating activity of phosphopeptone (admittedly "a

rather complicated mixture of phosphopeptides") for a number of lactobacilli has been studied by Agren (12). Stimulation is not observed with the free phosphorylated amino acids of phosphopeptone. Phosphopeptone is utilized as a source of phosphate by *L. casei*. The phosphorylated derivatives of serine, threonine, or glycine are utilized to some extent by *L. mesenteroides* as a source of these amino acids.

The rate of lactic acid formation in milk by various starter cultures in a standardized *in vitro* test was found by Anderson *et al.* (15) to be correlated with the peptide content of the milk. The correlation between rate of lactic acid production and peptide content was poor in a number of samples of milk from mastitic animals and from those in early or late stages of lactation.

PURINES, PYRIMIDINES AND NUCLEIC ACID DERIVATIVES

The pathway of nucleic acid biogenesis in bacteria is gradually being pieced together largely as a consequence of studies with mutants blocked at various stages of the synthetic pathway. The occurrence of 4-amino-5-imidazolecarboxamide in several wild-type and purine-requiring strains of *E. coli* grown without the presence of sulfonamides or aminopterin has been noted by Slotnick & Sevag (207). Difficulties encountered in characterizing trace amounts of this compound have left previous studies less decisive with respect to the occurrence of the amine in the absence of inhibitors. Love & Gots (130) found a purine-requiring mutant of *E. coli* that, in the presence of minimal amounts of hypoxanthine, produced a non-acetylatable, diazotizable amine that could be differentiated from 4-amino-5-imidazole carboxamide or its riboside by differences in its ultraviolet and diazo dye spectra. Evidence was presented that the new amine contains ribose and that it is converted to 4-amino-5-imidazolecarboxamide by another purine-requiring *E. coli* mutant. The new amine is presumed to be an aminoimidazole riboside (probably 2-aminoimidazoleriboside).

Evidence has been presented by Aaronson & Nathan (3) and Nathan *et al.* (157) tending to emphasize the significance of 4-amino-5-imidazolecarboxamide or its riboside or ribotide in nucleic acid synthesis at the expense of the corresponding derivative of 4-amino-5-imidazolecarboxamide. *Crithidia fasciculata* (two strains) was found to use the former free base but not the free base of the latter while for *Staphylococcus flavocyaneus* the former compound was twice as active. The following compounds were inactive as a purine source for *C. fasciculata*, *S. flavocyaneus*, and *Gaffkya homari*: 4-hydroxy-5-imidazolecarboxamide, imidazole-4,5-dicarboxylic acid, ethyl-4-amino-5-imidazolecarboxylate, 5-formamido-4-aminoimidazolecarboxamide, imidazole-4-carboxamide, 4-ureido-5-carboximidazole, formamidomalonomidamine, aminomalonomidamine, ureidosuccinic acid, and 2,4,5,6-tetraaminopyrimidine.

Utilization of the 2'- and 3'-nucleotides of adenine and guanine has been studied in a wild type and a purine- or histidine-requiring mutant of *E. coli* by Balis *et al.* (21). Adenine is utilized from the 3'- but not from the 2'-

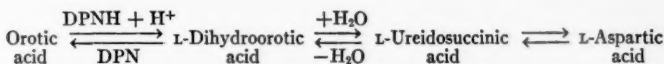
isomer. The phosphorus from the 3'- but not the 2'- is utilized nonspecifically, i.e. it appears in all the RNA nucleotides. These data indicate that incorporation of the purine moiety is therefore accompanied or preceded by liberation of the phosphate. On the other hand, in an extended study of the purine requirements of *S. flavocyanus* Aaronson found (2) that this organism utilizes adenosine 2', 3'- or 5'-phosphates equally well.

Griffin & Racker have observed (82) that the CO₂ requirement of several strains of *Neisseria gonorrhoeae* can be satisfied with oxalacetate and hypoxanthine. Tricarboxylic acid cycle intermediates substituted less effectively for the oxalacetate but hypoxanthine was specific among the purine derivatives studied.

Wood & Steers found (249) that a laboratory-developed, chloramphenicol-resistant strain of *Micrococcus pyogenes* var. *aureus* requires adenine plus either hypoxanthine or xanthine or guanine for growth. In cells grown on a medium containing adenine-8-C¹⁴ or guanine-8-C¹⁴ the label is found in the corresponding polynucleotide counterpart. With the parent strain guanine-8-C¹⁴ is found in both nucleic acid adenine and guanine, while adenine is found only in nucleic acid adenine. The resistant strain does not incorporate bicarbonate or formate into nucleic acid adenine or guanine when grown on a medium suboptimal with respect to guanine. The resistant strain is deficient, therefore, in one-carbon unit metabolism. The extent to which these differences are necessarily associated with chloramphenicol resistance deserves additional study.

Cohen & Barner (46) have observed that a thymine-requiring mutant of *E. coli* forms the adaptive enzyme xylose isomerase in the absence of thymine. When utilizing a carbon source for cytoplasmic growth in the absence of thymine the organism loses the power to multiply. Since in the absence of deoxyribonucleic acid synthesis (no thymine) there is induction and synthesis of an adaptive enzyme, it was concluded that the cytoplasm is the locus of this synthesis.

Mechanisms by which orotic acid is metabolized by aerobic bacteria have been studied by Reynolds *et al.* (188). The postulated sequence of of reactions is the exact reverse of those considered to exist in orotic acid biosynthesis in microorganisms:



Despite the fact that orotic acid appears to have more significance than the free pyrimidines in the synthesis of nucleic acids in animals and some bacteria (261), orotic acid does not satisfy the pyrimidine requirement of *Glaucoma scintillans* (115) and *E. coli* (strain 63-86) (153) to name only two species recently studied.

The synthesis of ureidosuccinic acid from "compound X" and aspartate by an enzyme of *E. coli* has been studied by Reichard & Hanshoff (187).

FATTY ACIDS

The liquid unsaturated fatty acid of *Agrobacterium* (*Phylomonas*) *tumefaciens* has been studied by Hofmann & Tausig (100). The acid, previously considered to be a 10- or 11-methylnonadecanoic acid was found to be identical with lactobacillic acid [probably one of the stereoisomers of cis-11,12-methyleneoctadecanoic acid (97, 98)].

Lactobacillic acid is absent from the fatty acids of a group C streptococcus [Hofmann & Tausig (99)]. The biotin activity of lactobacillic and related acids has been studied by Hofmann & Panos (98). The growth-promoting activity of the cyclopropane fatty acids for lactobacilli in a medium devoid of biotin depends on the presence of a chain of more than 11 carbon atoms. Two acids of shorter chain length, *trans*-dl-2,3-methylenonanoic and *trans*-dl-2,3-methyleneundecanoic acids, are inhibitors for several species.

Deibel & Niven (50) have observed that the apparent CO₂ requirement of the "minute" streptococci can be replaced by "tween 80" (polyoxyethylene derivative of sorbitan monooleate). The reciprocal relation was also established for two strains of lactobacilli where CO₂ replaces an apparent requirement for unsaturated fatty acid. For one streptococcal strain a water-soluble, ether insoluble, dialyzable component of yeast extract replaces the requirement for CO₂ or unsaturated fatty acid. High concentrations of pyruvic acid partially replace the requirement of the streptococcus for CO₂. One of the lactobacilli studied fixed large amounts of C¹⁴O₂ when grown with this gas in lieu of oleic acid. These studies are of significance also because of their possible relationship to the mechanism of action of biotin.

MISCELLANEOUS GROWTH FACTORS

Lactobacillus bifidus factors.—An unfortunate confusion has arisen in the literature concerning the nomenclature of factors promoting growth of various strains of *L. bifidus*. The unknown factor required for the growth of certain avian *L. bifidus* strains originally described by Shorb & Veltre (202) has been shown by Huhtanen (103) to consist of two components: pantetheine and a constituent of enzyme-hydrolyzed casein. Similar results were reported by Hendlin *et al.* (92) who showed that the peptide requirement is satisfied with certain arginine peptides or by high levels of arginine itself. The streptogenin requirement of *L. bifidus* studied by Roine *et al.* (191) also may be an arginine peptide.

The designation *L. bifidus* factor is most appropriately reserved for the factor(s) promoting growth of *L. bifidus* as encountered in the stools of infants in its native or fastidious form designated simply as *Lactobacillus bifidus* by Tomarelli *et al.* (226) or *Lactobacillus bifidus* var. *pennsylvanicus* by György & Rose (83).

A crystalline bifidus factor has been obtained from hog gastric mucin and identified as a galactose-acetylglucosamine [Tomarelli *et al.* (226)] more specifically 4-O-β-D-galactopyranosyl-N-acetyl-D-glucosamine [Zilliken *et al.* (271)]. From hydrolysates of human milk fractions a new compound with microbiological activity having the composition C₁₂H₂₁O₁₀N named gyna-

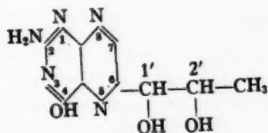
minic acid has been isolated [Zilliken *et al.* (268)]. The fractions from which the compound is derived also contain one or more components more active than gynaminic acid. A number of pure compounds are available with bifidus factor activity and include β -methyl-N-acetyl-D-glucosaminide (the corresponding α -glucoside is inactive) [Rose *et al.* (193)] and various alkyl N-acetyl- β -D-glucosaminides [Zilliken *et al.* (269)]. Evidence has also been presented [György & Rose (83)] for the existence of a supplementary factor concerned with the utilization of compounds with bifidus factor activity. The factor appears to be peptide-like in nature.

The enzymatic synthesis of 4-O- β -D-galactopyranosyl-N-acetyl-D-glucosamine from lactose and N-acetyl-D-glucosamine by intact cells of *L. bifidus* var. *pennsylvanicus* has been found by Zilliken *et al.* (270) to proceed without synthesis of the 6 isomer which is obtained in a 1/1 ratio with the 4 isomer when crude enzyme extracts are used. The 4 isomer which can be obtained crystalline in a yield of 5.4 per cent is active as "bifidus factor" while the 6 isomer is essentially inactive.

The whole "bifidus factor" problem has been briefly reviewed by György & Rose (84) and a summary of the microbiological activities of all chemically well defined materials studied has been summarized. It is concluded that the "true" bifidus factor has either still to be found or several related compounds may act as precursors or in a synergistic combination.

Biopterin (Crithidia Factor).—Growth of the insect trypanosomid *Crithidia fasciculata* has been shown by Nathan & Cowperthwaite (158) to occur in a synthetic medium only in the presence of large amounts of folic acid, or of smaller amounts of natural material, which were more active than could be explained on the basis of any probable folic acid content. The activity of natural material was ascribed to the existence of a new factor, Crithidia factor, probably related in structure or function to folic acid. Evidence cited by the authors favored the hypothesis that the Crithidia factor is a biological precursor of folic acid.

The isolation of the Crithidia factor from human urine and the characterization of the factor as 2-amino-4-hydroxy-6(1,2-dihydroxy-propyl)-pteridine have been described by Patterson *et al.* (165).



Biopterin (Crithidia factor) (2-amino-4-hydroxy-6 (1', 2'-dihydroxypropyl)-pteridine)

Additional microbiological studies with biopterin including an examination of the activity of other 2-amino-4-hydroxy-6-substituted pteridines are described by Broquist & Albrecht (36).

Independently the isolation of 2-amino-4-hydroxy-6(1', 2'-dihydroxy-propyl)-pteridine from *Drosophila* and synthesis of the compound was

described by Forrest & Mitchell (65). Viscontini *et al.* (231) also isolated what is probably the same pteridine from *Drosophila melanogaster*.

Glutamine.—The inhibitory effect of ethanol and certain other alcohols for *S. fecalis* has been shown by Ravel *et al.* (180) to be reversible by natural material. Using the antimetabolite reversing technique to follow concentration, the active component of hog liver was isolated and identified as glutamine. An accompanying paper (190) presents evidence that the oral administration of glutamine results in a decrease in the voluntary consumption of alcohol by rats. Glutamine administered parenterally was inactive.

Polyamines.—Conditions under which a putrescine requirement can be demonstrated with *Hemophilus parainfluenzae* have been studied in detail by Herbst *et al.* (93). Twenty compounds with some structural relationship to putrescine were examined. A number of derivatives promoted growth; others were putrescine antimetabolites. Putrescine has been observed to promote growth of an auxotrophic mutant of *Aspergillus nidulans* [Sneath (213)]. Related compounds were examined for activity.

Mager has observed (139) that *Neisseria perflava*, which requires putrescine or similar polyamine for growth on an amino acid medium but not on a commercial casein hydrolysate medium, does not require the amine when a suitable synthetic medium is supplemented with casein ash or sodium chloride. The factor is required, however, in the presence of tryptic digest of casein or salt-free acid hydrolysate. Growth promotion occurs when sodium chloride is replaced by relatively large amounts of potassium and lithium salts and by glucose and sucrose. These results indicate some degree of interrelationship between polyamines and osmotic agents. It is concluded that each can influence independently some essential cell function presumably having to do with permeability. Similar results were also obtained with *Pasteurella tularensis*.

Choline.—The occurrence of a sulfuric acid ester of choline $(CH_3)_3N^+CH_2CH_2OSO_3^-$ in the mycelium of *Penicillium chrysogenum* has been demonstrated by two groups [de Flines (63); Stevens & Vohra (218)]. The compound satisfies the choline or sulfur requirements of a cholineless mutant of *N. crassa*, but it is a poor precursor of penicillin sulfur compared with sodium sulfate.

Carnitine $(CH_3)_3N^+CH_2CHOHCH_2COO^-$ is inactive as a substitute for choline in the growth of cholineless *N. crassa* [Fraenkel *et al.* (68)].

Pentoses.—Camien & Dunn (42) have shown that *Lactobacillus gayonii* and a number of related species have a definite requirement for pentoses if they have been carried in media free of glucose (tomato juice). Adaptation to glucose utilization readily occurs. The pentoses are presumed to supply the energy source. It was concluded that in these organisms a pentose kinase is a constitutive enzyme while a hexose kinase is an adaptive one.

Rumen bacteria factors.—The activity of rumen contents in promoting growth of *Bacteroides succinogenes*, an organism indigenous to the rumen, has been traced by Bryant & Doetsch (41) to a synergistic effect of (a) a branched chain saturated fatty acid such as isobutyric, isovaleric, or α -methyl-n-

butyric acid, and (b) a straight chain saturated fatty acid from five to eight carbons in length. These results are in essential agreement with those of Bentley *et al.* (28) who found volatile fatty acids active in stimulating cellulose digestion by the mixed flora of the rumen in an *in vitro* or artificial rumen.

Hormones.—Growth of *Vibrio fetus*, an organism involved in abortions and lowered fertility in bovine and ovine species, is reported to be stimulated by progesterone, corpus luteum, and testosterone [Osborne & Bourdeau (164)]. Although the lack of specificity observed renders the findings open to suspicion, the results have interesting implications if they can be confirmed.

NEW AND UNKNOWN GROWTH FACTORS

Suggestive data are available pointing to the existence of additional unknown growth factors. Prince *et al.* (172) observed that six members of the genus *Flavobacterium* failed to grow in any medium simpler than nutrient broth. Brown & Binkley (40) could not grow certain smooth strains of *Sarcina lutea* on a synthetic medium adequate for certain rough strains. Wasserman *et al.* (241) isolated a number of anaerobic lactobacilli indigenous to the rumen that failed to grow on synthetic media. Potent sources of growth promoting materials were tomato juice, grass juice, distillers dried solubles, and bovine feces. MacLeod *et al.* (134) found a marine bacterium that could not be grown on synthetic medium. Eddy & White (56) endeavored without success to grow several strains of *Streptococcus pyogenes* in a synthetic medium containing galactose rather than glucose as the energy source. Uridine diphosphoglucose (galactowaldenase coenzyme) was essentially inactive. Some properties of the factor were described and it was suggested that the factor is derivable from nucleic acid. Smith (210) could not grow all strains of pleuropneumonia-like organisms on a synthetic medium, but progress was made in defining the requirements of less fastidious strains. Corn steep liquor was found by Kennedy *et al.* (113, 114) to be a powerful stimulant for *Lactobacillus casei*, *L. lactis* and a number of other lactobacilli. The basal medium contained protein digests so that the responsible material is probably not a peptide. The factor is highly polar, stable to acid and alkali on prolonged heating, dialyzable, and soluble in phenol. Certain impure proteolytic enzyme preparations have been shown by Kizer *et al.* (121) to stimulate growth of *L. casei* and *Streptococcus lactis*. Aqueous extracts of pancreas have been found by Sandine *et al.* (195) to be active in promoting growth of *L. casei* and *S. lactis*. Bioautography using a basal medium with or without a tryptic digest of casein showed the existence of two active areas. Since the area of activity could be observed in the presence of enzymatic digests of casein, it is presumed that these factors are not peptides.

In the interests of avoiding further confusion in the literature, this reviewer would like to see all future claims for the existence of new growth factors supported by the following data: (a) distribution of the factor in a variety of natural material expressed in suitable arbitrary units, (b) negative test of a substantial number of compounds of some biological significance,

(c) fractionation of natural material to an extent that the existence of a synergistic mixture of known compounds could be excluded.

INORGANIC ELEMENTS

Calcium.—Calcium ions stimulate the growth of *Lactobacillus fermenti* as they do with other lactobacilli previously studied [Yu & Sinnhuber (264)]. Response curves of *L. fermenti* to thiamine are longer and steeper with the inclusion of calcium in the basal medium. The calcium effect can be observed in natural as well as semi-synthetic media.

Copper.—The toxicity of this element for a vitamin B₁₂-methionine mutant of *E. coli* has been studied by Hine *et al.* (94). Growth on vitamin B₁₂ is markedly inhibited, but growth on methionine is little affected. It was concluded that copper inhibits some stage of methionine synthesis where vitamin B₁₂ is concerned.

Zinc.—Zinc has been shown to be essential for growth of *Fusarium vasinfectum* by Kalyanasundaram & Saraswathi-Devi (111). The fungus also produces a phytotoxin that is also antibacterial. The toxin is produced only within a narrow range of zinc concentration, namely 2–10 µg. of zinc per 25 ml. This level of zinc is, however, adequate for maximal growth.

Iron.—Healy *et al.* (91) have shown that in *N. crassa* an iron deficiency leads to a decrease in the following enzymes: catalase, peroxidase, succinic dehydrogenase, isocitric dehydrogenase, and dye oxidase. Glucose 6-phosphate dehydrogenase, diphosphopyridine nucleotidase and nitrate reductase are increased. A similar pattern of change occurs in cobalt toxicity.

NUTRITIVE REQUIREMENTS FOR PROCESSES OTHER THAN GROWTH

Tetanus toxin formation.—Pancreatic digests of casein, essential for the production of high titers of tetanus toxin, have been fractionated into acidic, basic, and neutral fractions by Mueller & Miller (156) employing ion exchange resins. A combination of all three fractions is as active as the original digest, but the omission of any one fraction (or suitable substitute) leads to little or no toxin production. The basic component can be replaced by the following peptides of histidine according to Miller (148): glyceryl histidine, 1- α -amino-N-butyl-L-histidine, carnosine and acetylhistidine. Anserine, DL-1-methyl histidine and DL-3-methyl histidine were found to be inactive.

Enzyme production.—The effect of serine or threonine additions to a growth medium on the deaminase activity of *E. coli* cells has been studied by Boyd & Lichstein (32). Certain quantitative considerations suggested the presence of two distinct systems catalyzing the deamination of serine and threonine but the data are not convincing.

Growth conditions essential for the production of the adaptive extracellular proteinase of *Streptococcus liquefaciens* were studied by Rabin *et al.* (178). Appropriate protein or amino acid combinations were effective.

E. coli and *A. aerogenes* cells harvested from carbohydrate-containing media were found by Boyd & Lichstein (33) to exhibit marked reduction of

tryptophanase activity. Hydrolyzed casein or appropriate amino acid mixtures added to carbohydrate grown resting cells restored activity.

Penicillinase production by *M. pyogenes* as influenced by the composition of the growth medium has been investigated by Bondi *et al.* (31). Stimulation of enzyme production was observed with tyrosine, cysteine, valine, and glutamic acid. Inhibition of enzyme production was obtained with isoleucine, threonine, serine, histidine, adenine, aspartic acid, glycine, and arginine. Enzyme production was increased with maltose instead of glucose as the carbohydrate. Purines seemed to depress enzyme synthesis. Although the object of the study appears to have been to develop a medium giving high yields of penicillinase, of equal significance is the possibility that a continuation of this study could lead to the development of inhibitors of penicillinase production with activity *in vivo*.

Riboflavin synthesis.—Smiley & Stone (209) have observed that the addition of propionic acid to commercial media consistently increases the yields of riboflavin obtained after growth of *Ashbya gossypii*. Propionic acid usually is considered as an inhibitor of microbial growth which in one case, at least, is reversed with lipoic acid [Stokstad *et al.* (220)].

The synthesis of riboflavin by *Eremothecium ashbyii* (closely related to *A. gossypii*) when grown in the presence of various compounds related in structure or function to adenine (which stimulates riboflavin synthesis by functioning as a moiety of the molecule) has been studied by Brown *et al.* (38). Azaxanthine and aminopterin inhibit synthesis. Folinic acid and to a lesser extent folic acid reverse the effect. Methyl adenine and 2-methyl xanthine stimulate synthesis significantly.

ANTIMETABOLITES

Amino acids.—Inhibition of a number of microbial species with canavanine and reversal of this inhibition with related amino acids has been studied by Walker (237). With *Torulopsis utilis* homoarginine equaled arginine as a reverser of the inhibitor. With *Chlorella vulgaris* homoarginine was an inhibitor of growth that was reversible with arginine or lysine. Subsequently Walker (238) demonstrated that homoarginine is a powerful inhibitor of *E. coli*. Reversal of inhibition is specific for arginine. A microbiological assay for arginine based on its reversal of homoarginine inhibition in this species was proposed. Such a method is 5,000 times more sensitive than a conventional assay with an arginineless mutant of *E. coli* and might have utility as an ultramicro assay for this amino acid. Certain lactobacilli that are resistant to inhibition by canavanine have been shown by Kihara *et al.* (116, 117) to contain an enzyme that hydrolyzes canavanine to homoserine and guanidine. Other resistant species, however, do not carry out this hydrolysis.

The synthesis of 2-cyclopentene-1-glycine has been described by Dennis *et al.* (53). The analogous cyclopentane derivative had been synthesized previously and shown to be an inhibitor of isoleucine utilization in *E. coli* [Harding & Shive (88)]. The newer analog is also a potent inhibitor of *E. coli*,

but in this instance reversal is obtained by a mixture of valine and isoleucine but not by either amino acid alone.

Previous studies of Ravel, Shive and collaborators have shown that glutamic acid inhibits the utilization of aspartic acid in reactions concerned with the biosynthesis of threonine in *Leuconostoc dextranicum* 8086 [Ravel *et al.* (182)] and with the biosynthesis of pyrimidines [Woods *et al.* (251)], threonine and lysine [Ravel *et al.* (185)] in *Lactobacillus arabinosus* 17-5. Similarly cysteic acid inhibits the biosynthesis of all three products of aspartic acid. Ravel *et al.* (181) have now found that glutamic acid and cysteic acid are synergistic inhibitors and that glutamic acid in contrast to cysteic acid does not prevent utilization of the precursors of aspartic acid, bicarbonate and oxalacetate. These data point to a sequence of inhibition with cysteic acid probably acting subsequent to glutamic acid.

On the other hand, aspartic acid is an antimetabolite of glutamic acid in *L. arabinosus* according to Ravel *et al.* (183). While this inhibition is reversed with glutamic acid, proline and citrulline (products of glutamic acid), glutamine is 50 to 100 times more active than glutamic acid. Reasons were presented for believing that glutamine is more active than glutamic acid because it is a better source of "active glutamyl" rather than because it functions as such in reversal.

Studies on the mechanism of action of the sulfonamides have established that the drug inhibits sequentially the synthesis of methionine, purines, serine, thymine, and valine. Alimchandani & Sreenivasan (13) have now shown with *E. coli* that at concentrations of sulfonamides in excess of those used in establishing the above sequence glycine and to a lesser extent threonine reverse the inhibition. These results indicate that glycine synthesis is yet another reaction indirectly influenced by sulfonamides.

Another instance of antimetabolite-metabolite relationships among the amino acids is norleucine inhibition of *Vibrio cholerae* and reversal with methionine and related compounds [Friedman *et al.* (69)].

Purines and pyrimidines.—Fries (70) has demonstrated that diaminopurine riboside is an antimetabolite for the growth of *Ophiostoma multiannulatum*. The compound is reversed by adenosine but not by adenine, guanine, guanosine, or diaminopurine. The latter compound which in itself promotes growth of guanineless mutants appears to have some inhibitory activity in conjunction with its riboside. It is concluded that the riboside cannot be an intermediate in nucleic acid biosynthesis by the fungus.

Twenty-five pyrimidine derivatives have been tested by Puleston *et al.* (177) for inhibitory activity against *S. fecalis* R. The following five compounds in decreasing order of activity are antimetabolites: 5-aminouracil, 2-thiouracil, 5-nitrouracil, 5-iodo-2-thiouracil, and 5-iodo-benzylthiouracil. As might be expected, uracil is effective as a reversing agent while thymine and cytosine also were generally active.

The preparation of 3-methylthymidine, 5-hydroxydeoxyuridine, and 5-bromodeoxyuridine from thymidine or deoxycytidine is described in detail

by Beltz & Visser (27). The deoxyuridine derivatives are powerful antimetabolites of a purine-requiring mutant of *E. coli* as demonstrated in this paper and in an earlier one (208). Additional microbiological studies are available in a preliminary note (26). The same group have described the preparation of the 5-chloro and 5-bromo derivatives of cytidine (71). These compounds are markedly inhibitory for a pyrimidine-requiring mutant of *N. crassa*.

The synthesis of a group of new compounds of uracil, 5-mercaptopuracil and related S-substituted derivatives, has been described by Bardos *et al.* (23). 5-Mercaptopuracil and its disulfide are potent inhibitors of *Lactobacillus leichmannii*. This inhibition is reversed competitively by thymine or thymidine but not by folic acid. The antimetabolite activity of these compounds and some previously known pyrimidine derivatives have been summarized by Bardos *et al.* (24). The results obtained permit the construction of a proposed scheme of DNA-thymidine biosynthesis in lactobacilli that is compatible with experimental findings.

The enzymatic synthesis of azathymidine (3,5-dioxo-6-methyl-1,2,4-triazine deoxyriboside) by a preparation from *S. fecalis* cells has been described by Prusoff (176). This compound is considerably more potent than azathymine as an inhibitor of lactobacilli. Possible mechanisms of action were discussed. It seems most probable that the compound is incorporated into cellular DNA. Presumably this abnormal DNA can prevent reproduction of the cell. The compound has interesting potentialities.

5'-Deoxyriboflavin or 6,7-dimethyl-9-1'-(5-deoxy-D-ribityl)-isoalloxazine has been synthesized by Shunk *et al.* (203). Preliminary data indicate that the compound is an antimetabolite of riboflavin for *L. casei*. It showed little or no activity against lymphosarcoma in animals on normal or riboflavin deficient diets.

SYMBIOSIS AND ANTAGONISM

The antagonism that exists between *A. aerogenes* and *B. subtilis* when grown on opposite sides of a cellophane membrane has been studied by Charlton (44). Competition for gaseous nutrients rather than any effect of direct contact or production of antibiotic material was found to explain the phenomenon.

Rose & György have observed (192) that occasional growth response curves of *Lactobacillus bifidus* var. *pennsylvanicus* to the "bifidus factor" (an oligosaccharide containing N-acetyl-D-glucosamine) were anomalous in that more growth and acid production occurred in the absence of the factor than in the presence of an intermediate quantity. The difficulty was traced to the occurrence of a mutation to the common *L. bifidus* with no "bifidus factor" requirement which was responsible for the growth in the absence of factor. *L. bifidus* var. *pennsylvanicus* during growth with adequate "bifidus factor" holds back the development of the mutant. The phenomenon was first observed in lactobacilli with tryptophan dependent and independent strains of *L. arabinosus* [Wright & Skeggs (260)]. The phenomenon has been

TABLE I

NUTRITIVE REQUIREMENTS OF MICROORGANISMS RECENTLY SUMMARIZED

MICROORGANISMS	REFERENCES
<i>Allomyces macrogynus</i>	(204)
<i>Bacillus anthracis</i>	(155)
<i>Bacillus</i> spp.	(175)
Bacteria (marine)	(134)
<i>Basidiomycetes</i> spp.	(107)
<i>Blastomyces dermatitidis</i>	(86, 87)
<i>Bordetella</i> spp.	(173)
<i>Brucella abortus</i>	(55, 57)
<i>Chlorella vulgaris</i>	(169)
<i>Chromobacterium</i> spp.	(129)
<i>Diplocarpon rosae</i>	(201)
<i>Flavobacterium</i> spp.	(172)
<i>Hemophilus pertussis</i>	(19, 106, 242)
<i>Labyrinthula</i> spp.	(232)
Lactobacilli (brewery)	(154)
Lactobacilli (oral)	(123)
<i>Lactobacillus bifidus</i>	(166)
<i>Lactobacillus leichmannii</i>	(263)
<i>Micrococcus lysodeikticus</i>	(25, 248)
<i>Pasteurella tularensis</i>	(62, 74, 140, 227, 228)
<i>Penicillium digitatum</i>	(59)
Pleuropneumonia-like organisms	(210)
<i>Propionibacterium</i> spp.	(60)
<i>Proteus</i> spp. (Providence group)	(174)
<i>Salmonella typhosa</i>	(102)
<i>Sarcina lutea</i>	(40)
<i>Schizosaccharomyces pombe</i>	(136)
<i>Sirolopidium zoophthorum</i>	(234)
Staphylococci	(81)
<i>Staphylococcus flavocyaneus</i>	(2)
<i>Stereum murrayi</i>	(189)
Streptococci ("minute")	(50, 51, 58)
<i>Streptococcus bovis</i>	(170, 171)
<i>Trichophyton tonsurans</i>	(225)
<i>Venturia inaequalis</i>	(66, 67)
<i>Vibrio cholerae</i>	(61)
<i>Vibrio fetus</i>	(164)
Yeasts (wine)	(247)

studied in great detail by Ryan & Schneider (194). The present studies show why the existence of a human milk factor-requiring *L. bifidus*, even though it is the predominant lactobacillus of the infant intestine, has escaped detection for so long. When plated out on media without a special "bifidus factor" supplement the ordinary *L. bifidus* is the predominant form obtained.

An ingenious apparatus for the study of symbiosis in microorganisms where single strains are separated from each other by a dialysis membrane has been described by Nurmikko (160). Utility of the apparatus was demonstrated with preliminary experiments where two or more strains each different in ability to synthesize different nutritive essentials could be grown in association.

MUTATIONS

Adelberg *et al.* (11) have tried without success to obtain mutants of *E. coli* and *Saccharomyces cerevisiae* with nutritional requirements for steroids. On the other hand, testosterone and progesterone have been reported to stimulate growth of *Vibrio fetus* (164) and a number of sterols promote growth of *Labyrinthula* species (233).

Goldstein & Smoot (78) have observed the existence of an unstable strain of *E. coli* that produces auxotrophic mutants during growth at a rate of approximately 1.5×10^{-3} per cell per division cycle in contrast to between 10^{-10} and 10^{-16} per cell per generation produced by usual strains of this organism. Strains dependent upon various vitamins, amino acids, purines, and pyrimidines, etc. were readily obtained. Unfortunately the auxotrophs are themselves inherently unstable and quickly revert back to the parent type.

NUTRITIVE REQUIREMENTS OF MISCELLANEOUS MICROORGANISMS

A number of recent studies have been concerned with comprehensive investigations on the specific nutritive requirements of various single species or groups of microorganisms. Most of these studies did not lead to the discovery of new growth factors or to the elucidation of metabolic relationships of fundamental importance and are consequently not discussed separately in the text. Such studies that have been noted are summarized in Table I.

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THE ANAEROBIC BACTERIA WITH SPECIAL REFERENCE TO THE GENUS *CLOSTRIDIUM*¹

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Since the literature concerning the anaerobic bacteria and their activities accumulates at the rate of 700 to 900 articles per year, obviously it will not be possible to review critically all pertinent literature nor even list a great many papers which deserve mention. As this topic has not been reviewed previously in this series [nor indeed elsewhere recently except one generally unavailable survey (147)], and in view of the mystery which at times appears to surround the topic of the growth of anaerobes, it is believed that more may be achieved if this summary is written for those unfamiliar with the field rather than for the specialists of the group. That the spore-bearing anaerobic organisms are important is attested by their association as casual agents of tetanus, botulism, gangrene, and a variety of animal diseases (237). In addition, certain species are active in the retting of flax, nitrogen fixation, the butyric acid and butyl alcohol-acetone fermentations, cellulose decomposition, and a wide variety of other biochemical reactions of interest and value. Thus this review will attempt to indicate some of the major reference source materials but, especially with regard to recent literature, will be limited to an indication of the trends of research rather than a detailed survey of the literature of the preceding year. Where possible recent longer papers or reviews will be cited to provide the reader with an entry to more of the literature both old and recent. Metabolic mechanisms, nutrition, sporulation and spore germination, and certain other topics will be excluded since reviews on these topics in the Annual Reviews and elsewhere generally have included information on the anaerobic bacteria. The Polish literature for 1945 to 1950 was reviewed by Borowski (25).

It may be well to indicate some of the basic literature with which all who attempt serious work with the anaerobes should be familiar. The discovery of the nonpathogenic, saccharolytic, spore-bearing anaerobe by Pasteur in 1863 should be considered the foundation of the work on anaerobic organisms. Von Hibler (270) is credited with the first general book, published in 1908. This was followed in 1918 by the monograph, *La Gangrène Gazeuse*, by Weinberg & Séguin (273) which evolved to *Les Microbes Anaérobies* by Weinberg, Nativelle & Prévot (272) in 1937. Meanwhile the urgency of the study of the war wound organisms occasioned the publication of special reports (38, 53, 283) concerning these organisms, and general reviews were published also in the various editions of the *Handbuch der pathogenen Mikroorganismen* and elsewhere. In 1948 the second edition of Prévot's (201)

¹ The survey of literature pertaining to this review was concluded in January, 1956.

classification manual appeared. Lebert & Tardieux (125) summarized the techniques for isolation and species determination used in Prévot's laboratory. Smith's recent monograph (237) will be found most valuable for practical information on anaerobic techniques as well as more than an introduction to the pathogenic species.

Three volumes (148, 150) of a detailed subject bibliography to the literature (excepting Slavic and Oriental) should prove valuable to those desiring a ready access to the original literature through 1939, the last year indexed. A second supplement, indexing literature through 1955, is contemplated, but publication will depend on availability of financial subsidy. Authors of articles on anaerobic bacteria can be of material assistance in this project by forwarding reprints of all publications, especially those in the more obscure journals, to the author of this review.

The author is asked often to supply references to general articles containing taxonomic surveys the results of which might allow the inexperienced to classify original isolations. Unfortunately, no such papers exist (at least in English) in which really comprehensive treatment is made of many of the species within the genus. Certain papers are, however, of considerable aid in this connection for frequently the "unknown" strain belongs to one of the toxigenic species, or the common associates of such, and those having need for this type of information would do well to study the critical paper of Hall (85). Although some of the techniques of the 1922 vintage have been changed and certain species names have been altered (Hall, believing oxygen relationships to be an inadequate character for generic distinction, preferred *Bacillus* to *Clostridium* for the anaerobic spore formers), this classic paper deserves constant reference. Equally valuable, particularly as the taxonomic key presented was supported by proposals of standard techniques including media formulae, is the paper of Spray (241). Reed & Orr (216) also present similar material with special reference to the gas gangrene anaerobes. For presumptive identification of a variety of species including many of the toxigenic types, egg yolk agar (149) appears to offer advantages. Details concerning the methods used in the French laboratories have been summarized (16, 125, 201). Simplified, to some extent, identification reaction schemes have been presented for use in field laboratories of military campaigns by Feeney (67) and others.

Cultivation techniques.—The obvious objective of providing culture conditions free of atmospheric oxygen may be achieved by a variety of methods. These are reviewed by Smith (237), Weinberg *et al.* (272), and in Leaflet III (4th edition, 1943) of *Manual of Methods of Pure Culture Study of Bacteria* (Biotech Publications, Geneva, New York, U.S.A.). For liquid cultures for general cultivation, the thioglycollate medium of Brewer (27) has proved most useful. Skerman (235) reveals the suitability of such media depends on presence of free sulphydryl groups. The Brewer jar has been modified (64) for use of any of the anaerobic culture methods based on the Laidlaw principle or with evacuation-replacement procedures and may be

used with manufactured, natural, or bottled gas or with carbon dioxide. The production of hydrogen by reaction of sodium borohydride in the presence of a catalyst in such jars has been suggested (28). For large scale work an apparatus has been devised utilizing helium (29). A closed chamber for use on a microscope stage permits observation of developing microcolonies (186). A glass slide-cover method has been developed for counting anaerobes (245). A single solution (glucose-sodium monohydrogen orthoarsenate-methylene blue) indicator for anaerobiosis appears useful (265) as does the single tube method (utilizing pyrogallol and alkali) for growth when it is desirable to follow the progress of the culture by turbidimetric procedures (133). A somewhat more complicated tube system (based on the Smith fermentation tube) was proposed by Wilson (278). Parker (183) suggested the use of iron wool wet with acidified copper sulfate as a means of achieving anaerobic conditions.

For enrichment cultivation a selective medium for the catalase negative bacteria including clostridia utilizes sorbic acid (59). Procedures for the enrichment of chromogenic types have been outlined (145). *Clostridium perfringens* may be isolated and presumptively identified using Nagler agar (containing serum or egg yolk) made selective by addition of neomycin (135) or sodium azide (109).

Aerobic growth of strict anaerobes.—The theory that strict anaerobes are unable to grow in the presence of atmospheric oxygen because of the production of lethal concentrations of hydrogen peroxide has been advanced by Gordon, Holman & McLeod (72). Much support has been given to this simple theory by the work of Holman (101) who demonstrated that the addition of a short column of catalase at the surface of a (tubed) shake culture allows growth of the anaerobe to the top of the column of medium and that gas appears in the layer containing catalase. Heated catalase and noncatalase containing liquids do not exert this effect. The absence of catalase from most anaerobic species and the correlation of production of catalase and resistance to hydrogen peroxide has long been known as indicated by Moland (163).

There have been frequent attempts to cultivate anaerobic organisms under aerobic conditions as exemplified by Dedic & Koch (45) who report aerobic growth of the single strain tested of *Clostridium tetani* (commonly considered as one of the more strictly anaerobic species) in broth upon the addition of 1 to 4 μg . Co^{++} per ml. of nutrient solution. Whether it will be possible to extend these results to other strains and other species remains to be seen. Important also will be the effect of Co^{++} on metabolic mechanisms in view of the report of Shankar & Bard (232) that a shift in fermentation type was observed in *C. perfringens* in the presence of Co^{++} .

Taxonomic studies.—Other than descriptions of proposed new species and a limited number of studies on small species groups there have been no major advances recently in the taxonomy of the clostridia except the proposal of Prévot (195, 201) for creation of new orders, families, and genera.

Unfortunately, to date, this system has not been accepted widely. It may be recalled that, beginning with the first edition, Bergey's *Manual* has used relationship to oxygen as the distinguishing characteristic between the aerobic genus *Bacillus* and the anaerobic genus *Clostridium* in the usual spore-forming eubacteria. Unfortunately the line of demarcation between the two groups is not a sharp one because of the characteristics of the gas-forming aerobic types (sometimes considered as a separate genus *Aerobacillus*) and the aerotolerant (microaerophilic) species usually included in the genus *Clostridium*. Clearly more attention needs to be given to the taxonomy of these transitional forms as well as to other species.

One of the most annoying difficulties which the novice will encounter in the literature concerning the anaerobic species is the abandon with which synonyms for a species continue in the literature. In this country and elsewhere *C. perfringens* and *C. welchii* are used interchangeably as are *C. novyi* and *C. oedematiens*. Most workers now use *C. septicum* though references to *Vibrio septique* continue to appear. *C. chauvoei* and *C. fesceri* refer to the same species. *C. sordelli* is reserved by some for toxic cultures of *C. bifermentans* though others use the latter name for both toxic and nontoxic strains having the general characteristics of the species. Possibly the most confusing interchange of names concerns the organisms producing the botulin toxin. According to the Bengtson (17) system the ovolytic strains should be designated *Clostridium paratubulinum* and the nonovolytic *Clostridium botulinum*. Although this system is used in the latest edition of Bergey's *Manual*, this distinction has been disregarded by many authors and thus confusion has arisen. It is most probable that all the proteolytic type A and B strains currently being studied in most laboratories should be designated as *C. paratubulinum* according to Bengtson. It has been proposed by Prévot (200) that *C. botulinum* be used for both the ovolytic and the nonovolytic types. Although this would reduce confusion it seems illogical in view of the marked differences in the physiological pattern of the two types. For those who are puzzled concerning the synonym situation most problems may be solved by reference to the species names in Section Id of the index bibliography (148, 150).

It may be recalled that Prévot is not the first to suggest subdivision of the organisms now included in the genus *Clostridium* of the Bergey *Manual*. Heller (95, 96) proposed a new family Clostridiaceae for the anaerobic rods which commonly were included then in the broad genus *Bacillus*. Two subfamilies, Putrificoideae and Clostridoideae, were proposed as were some 26 genera mostly named for prominent workers in anaerobic bacteriology; viz., *Omeliaskillus*, *Hiberillus*, *Robertsonillus*, *Nicolaerillus*, *Ermengemillus*, *Meyerillus*, *Weinbergillus*, etc. The Heller system was never widely adopted.

Since it may not be available to all, the outline of Prévot's system, together with brief descriptive details is as follows (195, 201):

Order Clostridiales Prévot (Sporulating gram positive and negative rods swollen at sporulation. Motile or nonmotile. Pigmentation sometimes present. Generally anaerobic but facultative forms possible).

Family I. Endosporaceae Prévot (Gram negative clostridia, spores central or subterminal).

Genus I. *Endosporus* Prévot (Motile).

Genus II. *Paraplectrum* Fischer (Nonmotile, encapsulated, spores subterminal).

Family II. Clostridiaceae Fischer emend. (Gram positive clostridia)

Genus I. *Inflabilis* Prévot (Nonmotile).

Genus II. *Welchia* Pribram (Nonmotile, encapsulated).

Genus III. *Clostridium* Prazmowski (Motile).

Order Plectridiales Prévot (Terminal spores).

Family I. Terminosporaceae Prévot (Gram negative plectridia).

Genus I. *Terminosporus* Prévot (Motile).

Genus II. *Caduceus* Prévot (Nonmotile).

Family II. Plectridiaceae Fischer emend. (Gram positive plectridia).

Genus I. *Plectridium* Fischer (Motile).

Genus II. *Acuiformis* (or *Palmula*) Prévot (Nonmotile).

C. parasporogenes was believed (justifiably) to be an invalid species (214). *C. thermocellum* (144), *C. hemolyticum* (238), and *C. tyrobutyricum* (30) have been redescribed. Both *C. oedematiens* and *C. septicum* produce a species specific hemolysin which may be neutralized by an appropriate antiserum (93). Tataki & Huet (255) claim that production of urease usually separates the toxic strains (*C. sordelli*) from the nontoxic strains of *C. bifementans*. The granulose reaction sets *C. beijerinckii* and *C. pasteurianum* apart from other butyrics (242). Hydrolysis of conjugated bile acids has been proposed (170) as a differential reaction.

Of the species recently described as new at least the following would appear important: the uric acid decomposing species *C. acidurici* and *C. cylindrosporum* (13); *Endosporus azotophagus*, claimed to be a new nitrogen fixing type (259); *C. cellobiophorus* which produces cellobiose as the principal product of digestion of cellulose (105); the chromogenic *C. aurantibutyricum* (98); *C. corallinum* (205), *C. saturni-rubrum* (199), and *Plectridium virens* (198); *C. kluyveri* which produces caproic acid from ethyl alcohol (15); *C. lactoacetophilum* which produces butyric acid, carbon dioxide, and hydrogen from lactate in presence of acetate (19); the new industrially important butyl alcohol producing *C. madisonii* (151); *C. propionicum* which produces propionic acid by fermentation of alanine (35a); the tartrate fermenting *C. tartarivorum* (160); the cellulose fermenting *C. thermocellulaseum* (61); *C. toanum* which produces butyl and isopropyl alcohols (9); *C. thermoaceticum*, a thermophilic type producing acetic acid by carbohydrate fermentation (68); and the unnamed retting clostridia of Lanigan (124).

The proposed scheme of Mandia (153 to 158) of antigenic types of the clostridia on the plan now in vogue for the *Salmonella* for serological identification of various species of clostridia awaits confirmation.

Hemagglutinating properties.—The agglutination of red blood cells by *C. septicum* has been claimed to be associated with the bacterial cells, not with the toxin (42). With the species producing botulinum toxin, there is a

lack of identity of hemagglutinating activity with toxicity in the Type A organism (123, 136), and the Types B, C, and D organisms are also concerned (122, 123, 246). In addition the phenomenon is also exhibited by *C. butyricum* (36), *C. perfringens* (79, 113, 159), and by nonspore-forming rods of Prévot's genus *Spherophorus* (254).

Bacteriophages for clostridia.—As judged by the papers included in the Spencer bibliography (240), Russian bacteriologists have isolated bacteriophages for the majority of the toxigenic clostridia, and some success is reported in the use of phage preparations as therapeutic agents. Phages for *C. perfringens* have been isolated in France by Guelin and associates (58, 75 to 78, 120), and in this country by Sames & McClung (229). The phage for the industrially important solvent producing *C. acetobutylicum* has been studied extensively by Kinoshita & Teramoto (116) and shown to be susceptible to assay by plaque count and dilution methods and to exhibit lysogenicity. Gold & Watson (71) characterized the phage for the related species *C. madisonii*, and Peri & Watson (189) have shown it to exhibit lysogenesis.

Toxins.—Possibly the most significant advance concerning the clostridia in the last two decades is reflected in the studies concerning the production, nature, and activity of the toxins elaborated by certain species. It is now clearly evident that some species produce a variety of toxins and often the various toxins require different conditions of growth and culture conditions for maximum production. In many instances the component toxins have been separated from the crude culture filtrates and characterized, and some have been purified chemically. Various reviews concerning the anaerobic toxins, the purification and enzymatic nature of some, and their role in the pathology of the disease process in which they are concerned are available and may be used as guides to the original literature (12, 139, 142, 174, 175, 182, 191, 212, 266, 267, 268, 280).

The most complex picture of a species producing a variety of different toxins yet presented is that now well established for *C. welchii* (*C. perfringens*) in which 12 soluble antigens have been detected. Fortunately, Oakley & Warrack (177) have outlined procedures for the routine typing of this species based on the detection of these antigens. Similar material for the three antigenic components in toxic filtrates of cultures of *C. histolyticum* were given by Oakley & Warrack (176) and for the six components for *C. oedematiens* (*C. novyi*) by Oakley, Warrack & Clarke (178). In addition to the isolation of the Type A and B botulin and the tetanus toxins (191, 237, 266), the important alpha toxin (lecithinase) of *C. perfringens* has been purified 130-fold on the basis of Lb activity per mg. of nondialyzable nitrogen (222); the theta toxin (223) and the hyaluronidase (11) have also been purified. The type D botulin toxin has been obtained in an electrophoretically homogenous but polydisperse state and shown to be even more toxic than the purified Type A toxin (275). In addition to the studies summarized in the reviews, recent reports concern the effect of *C. oedematiens* toxin on certain enzyme systems (130), the hemolysin (103), a cysteine-activated

proteinase (127), and collagenase (263) of *C. histolyticum*, the lecithinase of *C. bifermentans* toxin (129), the decomposition of tropomyosin by one of the *C. oedematiens* toxins (138), and the relationship of toxins of Type A *C. perfringens* to the pathogenesis of clostridial myonecrosis (70).

The gel diffusion technique is now being utilized in the study of the antigenic composition of the clostridial toxins (20, 80, 208). A series of reports concern the extraction of toxin from the cells or from cellular autolyzates of the tetanus (209, 252), botulinus (24, 112, 211), and gangrene organisms (210, 227, 228). For the production of high titer toxin growth of the organism in cellophane tubes immersed in an appropriate culture medium has been suggested for the tetanus and botulinus organisms (23, 69, 118, 248, 269, 275). A similar procedure has been used for production of cells of *C. chauvoei* for vaccine (247).

Mueller & Miller (167) found acid-labile components of tryptic digests of casein essential to tetanus toxin formation. They also discussed variable factors in the production of this toxin and have presented a formula suitable for large-scale production (166). Stone (251) suggested a modification (elimination of native protein) of the Mueller medium in which an adapted strain gave a high yield of toxin and from which toxoid of high purity was prepared.

Toxoids.—The effectiveness of tetanus toxoid as an immunizing agent is now firmly established by reports both from civilian, especially pediatric, and military practice (26, 74, 86, 88, 134, 137, 165, 190, 217, 231, 243, 244). The toxoid may be used alone or in combination with diphtheria and pertussis antigens (37, 56, 57, 108, 128, 230, 276). Effective toxoids have been prepared also against the chief agents of gas gangrene including *C. perfringens* (1, 5, 188, 249, 264), *C. novyi* (1, 5, 264, 271), and *C. septicum* (18, 219). Likewise, toxoids are available for Types A–D of the botulinus organisms (102, 213, 218, 248). Attempts to produce active immunity against botulinum toxin in mice by oral administration of toxoid was not successful (51) whereas intranasal administration of tetanus toxoid as a recall dose offers promise (279).

Occurrence of clostridia in soils.—Although the presence of anaerobic organisms in soils was noted soon after the recognition of such forms, it is interesting to note that Smith & Gardner (239) have established that *C. perfringens*, considered by some to be present in soil only as a result of fecal contamination, apparently does exist in soil in a heat-susceptible state with the inference that vegetative multiplication can occur there. The organisms producing the botulinum toxin have been found present in soil surveys made in this country in Illinois (110), Georgia (164), and central New York (184), and in Australia (54), India (185), England (83), and Sweden (65). These recent surveys reemphasize the need for eternal vigilance for proper methods of canning to prevent the possibility of toxic spoiled foods arising from heat resistant spores. The Type D organism has now been recovered from soil in Australia (55).

The occurrence of the toxigenic clostridia in soil, especially in relation to

military campaigns is reviewed by Zeissler & Rassfeld-Sternberg (284) and more recently by Smith (236). Many nonpathogenic species have been isolated from soil, particularly recently by Prévot, but space limitation prohibits listing of these reports.

Cellulose decomposition.—Although it has long been known that many organisms of various biologic groups are important in the breakdown of cellulose, the anaerobic organisms having this ability continue to receive attention particularly with regard to cultivation techniques by which pure cultures may be assured and to fermentation products. A good background to the problem of cellulose decomposition may be found in the review of Norman & Fuller (171); more recent reviews include those of Pochon (192) and Ostertag (181). McBee (143) concluded from a study of the available historical strains that most of the thermophilic spore-forming cultures now available could be considered [although as Enebo (62) points out there may be a taxonomic tangle attributable to probable impurity of cultures] as strains of *C. thermocellum* which has been redescribed (144). *C. thermocellulaseum* of Enebo (61) appears to be a distinct species on the basis of fermentation of monosaccharides. The associated noncellulose fermentor proposed preliminarily (61) as a new species, *C. thermobutyricum*, is now considered by Enebo (62) to be a strain of *C. thermosaccharolyticum*.

The mesophilic types include cocci [one of which has been named *Ruminococcus flavefaciens* by Sijpesteijn (233, 234)], nonspore-forming rods, and clostridia (104). Hungate (105a) proposed *Micromonospora propionici* as a new species for an anaerobic cellulose-decomposing actinomycete. Bryant & Doetsch (31) studied the fermentation products of *Bacteroides succinogenes*.

Flax retting and pectin fermentation.—Included in the group of organisms which are active agents of the natural (or to some extent controlled) retting of flax are a limited number of clostridia (226). Enebo (60) finds *C. felsineum* and *Granulobacter pectinovorum* Beijerinck and van Delden to be active in the hot-water retting of Swedish flax while Lanigan (124) reports four distinct types of retting clostridia active in Australian retting. *C. pectinovorum* Störmer and a new species were believed to predominate in natural retting while *C. felsineum* and a second new species were also present. In contrast, Allen (2) studying English flax has listed *C. tertium* as playing an active role in retting. Additional study will be needed on the strains so designated to determine their relationship to the *C. tertium* of wound origin. The four distinct types isolated from natural retting liquors in Israel by Hellinger (97) included *C. aurantibutyricum* [a new species of the butyl group described (98) earlier], and a new plectridial anaerobe *C. pectinovorum* variety *pseudoplectriforme*. *C. aurantibutyricum* and *C. felsineum* were shown to ferment pectin completely and actively ret flax straw, and the new *C. pectinovorum* var. *pseudoplectriforme* was moderately active whereas the typical *C. pectinovorum* showed little activity on pectin or in retting. Potter & McCoy (193) reported analyses of pectin and pectic acid fermentation by *C. felsineum* showed that their strains of *C. pectinovorum*, *C. butyricum*, and *C. tertium* failed to ferment pectin.

Food poisoning.—Recent studies have revealed that botulism is not the only disease of humans transmitted by foods in which anaerobes are involved. Two new diseases warrant mentioning. In Germany, from 1946 to 1949 several reports were made of a severe illness, sometimes fatal, in humans in which the characteristic picture involved a necrotic inflammation of areas of the intestine. It was suggested that the disease might be attributable to a bacterial toxin, and eventually the causal organism was identified as a type of *C. welchii* (*C. perfringens*). Various aspects of the disease, now termed *Darmbrand*, *enteritis necroticans*, or *hemorrhagic enteritis*, were reviewed by Hansen *et al.* (90). The organism involved was designated as a Type F of the species by Zeissler & Rassfeld-Sternberg (284), chiefly on the basis of heat resistance of the spores. The toxin pattern of the Type F organisms has been examined (173) and, though similar to Types B and C, appears to justify the creation of the new type. The heat resistance of the spores explains the transmission of the organism by the eating of insufficiently sterilized canned food. Cases were reported following eating of canned rabbit meat (81). Spores of the Type F organism have been isolated from one-sixth of 108 stool specimens tested from persons not suffering from the disease (82), and thus the possibility of human carriers must be envisioned. Dieckmann (46) revealed that two of 63 cultures of this species in Zeissler's collection were Type F strains (on basis of heat resistance); both had been recovered from war wounds.

The same species has been implicated in an acute gastro-enteritis characterized by abdominal pain and diarrhoea without vomiting. The British outbreaks were studied carefully by Hobbs *et al.* (99) who showed the strains of *C. welchii* to be feebly toxigenic, generally corresponding to Type A and producing heat resistant spores. The characteristic mild food poisoning was reproduced in human volunteers by ingestion of cultures isolated from contaminated meat. Strains of this type were also isolated from the feces of pigs, rats, mice, and cattle and from market samples of pork, beef, and veal. A similar poisoning attributed to this species had been reported from America by McClung (146) although Dack *et al.* (41) were unable to produce illness in volunteers by feeding cultures from the American outbreaks. Österling (180) was able to reproduce the disease upon feeding volunteers with strains isolated from Swedish outbreaks.

Strains of Type D have now been isolated (119) from a human with evidence of colonic disturbance; this type was previously known as the agent of enterotoxemia of sheep and goats and pulpy kidney of lambs.

Much information of value on the role of the anaerobic bacteria in the spoilage of "semi-preserved" foods may be found in the several papers of the 1st *Symposium International de Bacteriologie Alimentaire* published in the 1955 volume of *Annales de l'Institut de Lille* and in the reports of Butiaux (32, 33).

Botulism.—In view of the extremely potent toxin produced by the botulinus organisms, there is need for eternal vigilance with respect to observing the proper precautions in the preparation of food intended for human use

(47, 161). Process ripened soft cheese must now be added to the list of possible botulinogenic foods (162). Of more than passing interest is the number of reports of Type E botulism from the Pacific Northwest (48, 49, 50, 52), Denmark (187), and from France (202, 203, 204). Dolman has reviewed the complete literature (48). The Type E organism was isolated from soil by Dolman & Kerr (52) and from soil and sea mud by Pedersen (187). The first reported cases of human botulism in Australia were traced to canned beetroot (73).

Gas gangrene and tetanus.—Many of the reports relating to wound bacteriology offer primary material concerning gas gangrene as do the discussions of the toxins produced by the agents of this disease, but some will find useful the general summaries by Altemeier & Furste (6), Reed & Orr (215), Smith (236), and Oakley (172). Similar general material is available for tetanus (221, 281).

Wound bacteriology.—It is accepted generally that the bacterial flora of contaminated wounds often include anaerobes as well as aerobes. Although the chief gas-gangrene anaerobes are *C. perfringens*, *C. oedematiens*, and *C. septicum*, other clostridia (in addition to streptococci) of concern may be present. These may include *C. histolyticum*, *C. bifermentans* (the toxic strains of this species group are designated *C. sordelli* by some), *C. tertium*, *C. multifementans*, *C. sporogenes*, and others. The possibility of the presence of *C. tetani* must be remembered, and now we must add the botulinus organism to the list in view of the reports of cases of botulism arising as a complication of wound infection (43, 87, 261) and Hall's earlier report (84) of the recovery of both Type A and the Type B organisms from wounds in patients who did not develop botulism. In this regard Keppie's report (114) that twenty million botulinum spores freed of all toxin are necessary to cause death of mice by intramuscular injection is of interest.

The determination of the exact organisms present in a contaminated wound is not an easy task and normally requires so much time for purification and species identification that the resulting information may be of little value to the surgeon in view of the possibility of the rapidity of the developing disease process. Nevertheless studies are important for the comparative picture, with differing conditions, of the spectrum of organisms likely to occur, and it is significant that surveys of material from the recent military campaigns (and also of civilian origin) have confirmed to a great extent the picture obtained from similar surveys of World War I wounds. This material is available in many summaries such as those of Altemeier (3, 4, 7), Smith (236), Zeissler (282, 283), and other reports (38, 53, 207, 224).

Practical procedures for the examination of wounds and general species identification tests have been presented by Tulloch (262), Hayward (92), Feeney (67), and Reed & Orr (215, 216). Recent examples of the reports on the bacteriology of war wounds includes material from the Philippine Islands (39, 66), the Pacific area (168), the Carelian Isthmus (225), Tripolitania and Tunisia (140), the Middle East (141), Italy (250), and Korea (132, 253).

Spencer's important annotated bibliography (240) summarizes the Russian literature on this topic.

Antibiotics.—The sensitivity of anaerobic bacteria to the common antibiotics is of interest for several reasons of importance. It has been proposed that, prior to canning, addition to certain foods of an antibiotic which would inhibit the spoilage bacteria might allow marked reduction of the process time required for canning. Cameron & Bohrer (34) point out the factors necessary to the acceptance of such a procedure. These would include laboratory proof of ability of antibiotics to destroy spores of the botulinus and the saprophytic spoilage bacteria, heat stability of the antibiotic, successful pilot scale inoculated experimental packs and public health and industrial acceptance of such a procedure. The comprehensive review of Campbell & O'Brien (35) reveals that while there appears promise of use of antibiotics in the prevention of spoilage in fresh foods it is unlikely that there will soon be acceptance of the substitution of an antibiotic for heat processing of canned foods. Of importance in this regard is the fact that severely heated spores of typical spoilage organisms are more sensitive to subtilin, the antibiotic most commonly proposed for use in canned foods (179). The action seems to be sporostatic rather than sporocidal (277). The possible use of antibiotics in relation to preservation of meats was reviewed by Ingram & Barnes (107).

The use of antibiotics at the time of surgery may be of aid in reducing or eliminating anaerobic infections. This is discussed by Pulaski (206, 207), and Dearing & Heilman (44) recommend the administration of aureomycin to the patient prior to surgery (see also Jawetz, this Review). With regard to prophylaxis of experimental tetanus infections Taylor & Novak (257) found chlortetracycline (aureomycin), penicillin, and oxytetracycline (terramycin) to be effective in reducing mortalities; penicillin was effective similarly for infections with *C. perfringens*, but with *C. septicum*, although time of death was delayed, reduction in mortality was not evident (256). In other experiments antitoxin was more effective than antibiotics (258). Several reports concern the bacterial (anaerobic) spectrum of various antibiotics including penicillin (8, 117, 131, 169), chlortetracycline (8, 94, 126, 131, 274), oxytetracycline (8, 117, 126, 131, 167, 274), chloramphenicol (8, 117, 131, 169, 274), carbomycin (274), erythromycin (94, 274), polymyxin B (131), and bacitracin (131).

Nonsporulating anaerobic bacteria.—Although the present status of the information concerning the spore-forming anaerobic bacteria leaves much to be desired with regard to many topics, the situation with respect to the nonspore-forming types is less bright (237). Although as the title indicates this review has been concerned chiefly with the spore-bearing species, it may help to indicate some of the reference material concerning the nonspore-forming anaerobic bacilli and cocci which has appeared since the general review of Dack (40). Erikson's review (63) should be mentioned, and Rosebury (220) summarized the confusing literature on the parasitic actinomycetes including *Actinomyces israeli* and other filamentous microorganisms

of the mouth. Kasai (111) presented information on culture medium requirements for the growth and isolation of such organisms. Hvid-Hansen (106) isolated *A. israeli* from ground-water, and Kimball *et al.* (115) found *Actinomyces bovis* to be the agent of bovine orchitis. Hoffman (100) reviewed the biology of the oral spirochetes, some of which are claimed to be strictly anaerobic and all appear to have exacting culture requirements. Thjötta & Jonsen (260) have restudied *Bacteroides*, and McVay & Sprunt (152) give detailed information on *Bacteroides funduliformis* isolated from human infections. Bain (10) isolated such organisms from the joint fluid in a case of arthritis in a foal. Bøe's (21) monograph on *Fusobacterium* includes historical material as well as experimental studies of this important genus, and Lahelle (121) studied the relationship of this group to the proposed genus *Necrobacterium*. The gram-negative rods were studied by Hansen (89). Hungate (104) reviewed the cellulolytic species.

Prévot proposed classification of the anaerobic nonspore-forming straight rods (194), vibrios (196), and the actinomycetes (197), and Bonomi (22) considered classification of the latter group, particularly of the Wolf-Israel type. Hare *et al.* (91) perhaps have presented the best information on the anaerobic cocci. Barker & Haas (14) proposed *Butyribacterium* as a new genus for gram-positive nonsporulating rods chiefly of intestinal origin.

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NUTRITION OF PROTOZOA^{1,2}

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The last review of protozoan nutrition which appeared in this publication was in 1951 (1). Since that time three volumes (2, 3, 4) have been published which treat in a detailed way the developments in the field of protozoan nutrition up to 1955. Because of the availability of these detailed discussions, this review will focus on some of the works of the past five years which appear to the writer as important in indicating the major trends in the field, and also on those studies which have been reported during the past year.

Progress in protozoan nutrition has been slow, partly because of the very complex nutritive requirements of many of the species. Our knowledge in this field is still scanty when compared to bacterial nutrition, but much has been accomplished in recent years. Real progress is made when an investigator is able to culture a protozoan in the absence of other living organisms. Once this has been realized it is possible to start the analysis of the medium and work toward a chemically defined medium. It is only when this has been attained that a complete knowledge of the nutritive requirements of a species is available. At the time of the last review all of the nutritive requirements of the ciliate, *Tetrahymena*, had been determined except one unknown substance. The nature of this substance has now been determined and will be described below.

The review is divided into four sections. The first section will be concerned with the nutrition of free-living ciliates, the second with the nutrition of free-living flagellates, and the third with free-living amoebae. These sections will be followed by one which will consider some of the work which has been done on the nutrition of parasitic and symbiotic species.

CILIATES

Tetrahymena.—In 1951, Kidder (1) stated that more work had been done on the nutrition of *Tetrahymena* in the preceding five years than on any other protozoan species. This organism has continued to be a favorite in protozoan researches generally. With the identification of the last unknown in a long list of nutritive requirements, much of the recent work has been focussed on the metabolism of the different required substances. Seaman (4) has given a rather complete account of these studies.

A complete list of the nutritional requirements of *Tetrahymena pyriformis*

¹ The survey of literature pertaining to this review was completed in January, 1956.

² The following abbreviations are used: CoA (coenzyme A); DNA (deoxyribonucleic acid); NDF (nondialyzable fraction); p.y.j. (pressed yeast juice); RNA (ribonucleic acid).

has been given by Kidder (5) as follows. Ten amino acids: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine; six vitamins: thiamine, riboflavin, pantothenic acid, pyridoxal or pyridoxamine, nicotinic acid or nicotinamide, pteroylglutamic acid, and thioctic acid; two nucleic acid derivatives: guanine and uracil or cytidine; and inorganic substances including phosphate, magnesium, potassium, iron, copper, zinc, cobalt, calcium, and manganese. Kidder points out that the data on the requirements for zinc, cobalt, calcium and manganese are still incomplete.

The last of the requirements to be identified was thioctic acid and a number of investigators were involved in the identification of this vitamin. In 1941, Dewey (6) showed that *Tetrahymena* could be grown in a casein medium only when it was supplemented with crude fractions of natural materials. In 1944 (7) Dewey proposed the term Factor I for the material precipitated from water extracts of natural substances and Factor II for the material in the filtrate. Both factors were necessary for growth, even in the presence of the known B-vitamins. In 1945, Kidder & Dewey (8) showed that Factor I could be replaced by a purine and folic acid. At this time, with the exception of Factor II, all of the growth factors for *Tetrahymena* had been determined. Factor II was supplying the thioctic acid. Two related observations were made next. Guirard, Snell & Williams (9) reported that acetate could be replaced in the medium used for cultivation of *Lactobacillus casei* by minute quantities of yeast extract. The active component in the yeast extract was called "the acetate replacing factor." In 1948 O'Kane & Gunsalus (10) reported that a strain of *Streptococcus faecalis* failed to oxidize pyruvate unless a factor from yeast was present. They called this factor the "pyruvate oxidation factor."

In 1949 Stokstad *et al.* (11) were able to concentrate the active material in Factor II for *Tetrahymena* and proposed the term protogen for this factor until a more appropriate name, based on chemical structure, could be assigned. In the same year Snell & Broquist (12) presented evidence which indicated that protogen, the acetate replacing factor and the pyruvate oxidation factor were identical. In 1951, Reed *et al.* (13) succeeded in crystallizing the factor and proposed the name α -lipoic acid for the crystalline material. Later the same year the Lederle group also crystallized active material. In 1952 they established the structure of protogen and proposed the name thioctic acid for it (14). In the current literature both names, thioctic acid and lipoic acid, are being used.

In contrast to the complete replacement of thioctic acid by acetate for growth of *L. casei*, acetate only spares the thioctic acid requirement for growth of *Tetrahymena* (15, 20). It was thus obvious that reactions besides the formation of acetate from pyruvate are dependent upon thioctic acid. In 1952 Dewey & Kidder (16, 21), working with thioctic acid deficient cultures of *Tetrahymena*, found an accumulation of α -ketoglutarate as well as pyruvate, which indicates that this factor is necessary for the oxidative

decarboxylation of the two keto acids, pyruvic and α -ketoglutaric. Seaman (17, 18, 19) has shown that thioctic acid is essential for the transfer of acyl groups to CoA. By growing his organisms at a low pH, he was able to show an increased sparing action by the use of a combination of succinate and citrate with acetate.

Elliott (22) has stated that serine is an absolute requirement for the growth of *T. pyriformis* strain E. Kidder & Dewey (23) have found that, although serine is required for optimum growth of strain W under ordinary cultural conditions, strain W can synthesize serine provided: (a) alanine, which inhibits the utilization of both exogenous and endogenous serine, is omitted from the medium; (b) folic acid is present in high concentrations; and (c) thioctic acid is increased tenfold over the amount required for optimum growth in the presence of serine. They state that under these conditions serine is synthesized by *Tetrahymena* by condensing glycine with a one carbon fragment derived most efficiently from glucose or other fermentable carbohydrate. They also point out that the one carbon fragment is not formic acid, or the methyl carbon of methionine, sarcosine, betaine, or choline, as all of these compounds are inactive. Elliott & Hayes (24) have recently examined 77 sexually active strains of *T. pyriformis* from Mexico, Panama and Colombia for nutritional requirements in axenic cultures. They found that six of these strains grow without serine. Their preliminary results indicate that, when one of these six strains is crossed with a serine-requiring strain, the character follows a Mendelian pattern of inheritance.

In a study of the factors affecting the requirement of *T. pyriformis* for folic acid, Dewey & Kidder (25) found that thymine or thymidine, glycine, *p*-aminobenzoic acid, creatin, and vitamin B₁₂ diminish the requirement for folic acid. Pteric acid, *p*-aminobenzoyleglutamic acid, 5-methylcytosine, *p*-hydroxybenzoic acid, carbamylaspartic acid, and ribonucleic acid have no effect on the requirement, while cholesterol and cortisone increase the requirement.

Hutner *et al.* (26), Kidder *et al.* (27), and Slater (28) have discussed the technical difficulties involved in evaluating the roles of metals in the metabolism of microorganisms. As mentioned above, Kidder has indicated that the data on the requirements for zinc, cobalt, calcium, and manganese are incomplete. Slater (29) has presented evidence that cobalt is a requirement for *Tetrahymena*. Hall (30) has studied the effects of certain metals on the growth of *T. pyriformis* in a peptone medium with the help of chelating agents and cation exchangers. He states that it is difficult to attribute the effect of added Ca to displacement of another metal without assuming that there is some essential metal occupying a position intermediate between Ca and Mg in the stability series of ethylenediaminetetraacetic acid tetrasodium salt complexes. He adds that until such an essential metal is recognized, it seems impossible to avoid the conclusion that Ca plays a role of some importance in the metabolism of *T. pyriformis*.

Kidder *et al.* (31) have reinvestigated the effect of non-ionic detergents

on *Tetrahymena*. It was found that certain oleic acid-containing detergents were stimulatory during early growth phases. The organisms appeared to break down the detergent molecule and to store large quantities of the fatty acid. Cell numbers at the end of a four-day growth period were the same for Tween-grown organisms and the controls, as was the cellular nitrogen content. The turbidimetric readings were much higher in the Tween-containing cultures because of the larger and more fragile lipid-filled cells. They do not recommend the use of detergents when *Tetrahymena* is to be used in experiments when cells must undergo manipulative procedures.

Holz & Tompson (32) have recently established *Tetrahymena rostrata* in axenic culture. Preliminary studies indicate that it will grow and reproduce in a medium containing a mixture of amino acids, "vitamin-free" casein, glucose, B-vitamins, nucleic acid components, and mineral salts. The absolute requirements have not been determined.

Paramecium.—The first axenic cultures of *Paramecium* were established in 1942 by Johnson & Baker (33) in a medium composed of proteose-peptone and a filter-sterilized pressed yeast juice (p.y.j.). In 1945 Johnson & Tatum (34) were able to show that two separate fractions of the p.y.j. were essential for the growth of *Paramecium multimicronucleatum*. One fraction was heat-labile, nondialyzable, and could be precipitated at three-fourths saturation with ammonium sulfate; the other was heat-stable and remained water soluble after autoclaving. In attempting the elucidation of the nature of the heat-labile component, Johnson (35) was able to substitute hydrolyzed nucleic acid, and still later guanylic and cytidylic acids for this yeast fraction. This may mean that *Paramecium* lacks the ability to free nucleotides from nucleic acid and that the natural ribonuclease, or deoxyribonuclease of the yeast or both were doing this in the heat-labile fraction. The medium then consisted of guanylic and cytidylic acids, autoclaved p.y.j. supernatant fluid, and proteose-peptone.

In recent studies, Johnson & Miller (36) have used a more convenient method for the preparation of the essential material from yeast. Using Fleischmann's "active dry yeast" a preparation was obtained which supported better growth in proteose-peptone than did the p.y.j. fraction. By dialysis this preparation was separated into two required fractions: the dialysate and the nondialyzable fraction (N.D.F.). Eighty per cent of the solids were found in the dialysate in the course of an exhaustive dialysis, and it was found that a mixture of eight B-vitamins completely replaced the dialysate fraction. The N.D.F. contains polysaccharides, has a low Kjeldahl nitrogen content, and an ash value of 8 per cent.

In earlier work (35) it had not been possible to obtain growth of *Paramecium* in the *Tetrahymena* medium with the yeast fraction added. Johnson (37) found that Tween, which had been regularly included in the *Tetrahymena* medium, was toxic for *Paramecium*. In 1953 van Wagendonck *et al.* (38) found that a sterol was required for the growth of *Paramecium aurelia*. Using a modified *Tetrahymena* medium, minus Tween and with stigmasterol

added, it was possible to obtain good growth of *Paramecium* with small amounts of N.D.F. added (36). The replacement of proteose-peptone with a mixture of amino acids gave a medium defined for all components except N.D.F. and permitted the determination of several nutritional requirements for *P. multimicronucleatum*. It was readily shown that phosphates are required in the medium, for no growth occurs in their absence. There is an absolute sterol requirement which may be satisfied by stigmasterol. Growth fell to zero in the seventh transfer upon the omission of stigmasterol. The optimum concentration of stigmasterol in the medium used was 2 μ g. per ml. It was also found that acetate is essential for growth. Growth is markedly reduced with the omission of acetate even when the N.D.F. was used at a maximum concentration. Of several carbon sources tested none would completely replace the acetate. The mixture of B-vitamins which was used to replace the yeast dialysate included folic acid, riboflavin, thiamine, pantothenate, nicotinamide, pyridoxal, choline, and biotin. By the single omission technique it was readily possible to show that folic acid, riboflavin and thiamine are required, even when using the N.D.F. at a level of 1 mg. per ml. When the concentration of N.D.F. was reduced by one-half, growth in the tubes minus pantothenate failed after five serial subcultures. In those tubes minus nicotinamide and pyridoxal growth was significantly reduced, which strongly indicates they are required. No requirement for, or stimulatory effect of, choline, biotin, B₁₂, and thiocetic acid have been demonstrated up to the present time. The nature of the requirements for nucleic acid derivatives in this more defined medium has not been determined.

Since the N.D.F. has a low nitrogen content, Johnson & Miller (39) were able to analyze for specific amino acid needs by the single omission method. Absolute requirements were found for tyrosine, phenylalanine, tryptophan, methionine, threonine, isoleucine, leucine, histidine, valine, and serine. The omission of lysine, arginine, proline, alanine, glutamic acid, aspartic acid, and glycine resulted in a reduction of growth.

P. aurelia was first established in axenic culture in 1949 by van Wagten-donk & Hackett (40). Their medium consisted of yeast extract (Basamin-Busch), lettuce extract, and *Aerobacter aerogenes*. *A. aerogenes* was inoculated into a lettuce extract and autoclaved after 24 hours incubation. The yeast extract and lettuce infusion were then mixed aseptically in a ratio of one to one. All three components were essential for growth; however, the growth was not as high as in bacterized medium. Subsequently, van Wagten-donk, Miller & Conner (41) developed a medium, composed of yeast extract, proteose-peptone, and a steroid from plant sources, which gave excellent growth. All three components were essential. Exhaustive dialysis of the yeast extract freed it from those water-soluble vitamins which are not bound to proteins, and they were able to demonstrate that folic acid, riboflavin, and thiamine are essential for *P. aurelia* (38). Folic acid was required in a concentration higher than is required for most organisms. It could not be replaced by *p*-aminobenzoic acid.

Using this medium it was possible to determine the nature of the steroid from plant sources. In 1953 Conner, van Wagtenonk & Miller (42) isolated the factor from lemon juice, and on the basis of its infra-red spectrum tentatively identified it as β -sitosterol. Following this many steroids were tested. In 1955 Conner & van Wagtenonk (43) reported that steroids having the cholesterol configuration were inactive. The following were active in supporting growth: β - and γ -sitosterol, fucosterol, brassicasterol, stigmasterol and Δ 4, 22-stigmastadienone. Esterification of the 3-hydroxyl group decreased the activity. The presence of more than one double bond in the ring system inactivates the molecule, as do the more drastic changes in the side chain, as found in diosgenin, digoxigenin, progesterone, estrone and methyl cholate. Oxidation of the ring system destroys activity. The specificity of these requirements suggests that the steroid functions as an essential metabolite for this organism.

van Wagtenonk (44) compares the growth-promoting activity of various steroids and points out that the specificity studies by Cailleau (45) on the steroid requirements of *Trichomonas columbae*, the studies by Vishniac & Watson (46) on those of *Labyrinthula vitellina*, the studies by van Wagtenonk & Wulzen (47) on the steroid requirements of guinea pigs, as well as those on *P. aurelia*, indicate a true metabolic requirement for steroids that is more general than was hitherto suspected.

Quite recently Miller & van Wagtenonk (48), working with five strains of *P. aurelia*, were able to partially analyze the nitrogen requirements of this species. In this study the essential material from yeast was present in an exhaustively dialyzed yeast extract of Fleischmann's "active dry" yeast. This preparation was used later by Johnson & Miller in studies on *P. multi-micronucleatum* as described above (36, 39). When this nondialyzable yeast fraction (N.D.F.) was added to a medium containing salts, amino acids, vitamins, sodium acetate, sodium pyruvate, and stigmasterol, good growth was obtained. The sterol requirement for strain 57.7 was described above, and in 1955 Conner (49) reported that stock 47.8 also requires sterol. All five stocks were established in an amino acid medium. In a detailed study on stock 47.8, using low concentrations of N.D.F., they found an absolute requirement for the following amino acids: tryptophan, methionine, threonine, isoleucine, leucine, lysine, histidine, arginine, serine, and both phenylalanine and tyrosine. There was also evidence of a need for an exogenous source of valine, proline, and glycine in their studies.

It had been shown earlier (38) that stock 51.7, even in the presence of proteose-peptone, requires folic acid, riboflavin, and thiamine. In this later study the need for these three vitamins was easily shown. Also, when the concentration of N.D.F. was reduced, it was possible to show a need for pantothenate and nicotinamide, with an indication that pyridoxal may be a requirement.

Hamilton (50) had indicated that stock 51.7 of *P. aurelia*, growing in a

bacterized medium, utilizes adenine, guanine and 2,6-diaminopurine for polynucleotide synthesis. Miller & van Wagtendonk were unable to show clear-cut requirements for nucleic acid derivatives in this study, probably due to the presence of these compounds in the N.D.F. However, in the course of testing several nucleic acid derivatives, they found a marked inhibition by 2,6-diaminopurine, even at levels of 1 to 5 $\mu\text{g.}$ per ml., for both stock 51.7 and stock 47.8. This difference in results again points to the need for using axenic cultures in determining nutritional needs and in analyzing protozoan metabolism. Although no specific differences in the nutritional requirements of the five different stocks were noted, the different stocks showed definite differences in growth rate. The growth rate in a given medium always followed this pattern: stock 47 > stock 32 > stock 51 > stock 29. The reason for the differences in growth rate remain unknown, but Miller & van Wagtendonk point out that the average rate of growth of the stocks corresponds roughly to the increase in chromosome number as reported by Dippe (51) in 1953.

On comparing the nutritional requirements of *P. aurelia* and *P. multimicronucleatum* it is clear that they share a sterol requirement. It also appears that their vitamin requirements may be the same. Although a few differences in the amino acid requirements were reported, these may be due to variations in the unknown N.D.F. Somewhat different results are obtained with different batches of N.D.F., and until this is replaced with a known substance, or substances, the complete list of requirements for the species of *Paramecium* cannot be given. It seems very probable that the presence of N.D.F. in the medium makes impossible the determination of the nucleic acid requirement. It would appear that both species are unable to oxidize phenylalanine to tyrosine, since an exogenous source of both amino acids is essential for continued growth. Most animals can meet their tyrosine needs when supplied with a source of phenylalanine, although the reverse reaction apparently does not occur. Tyrosine will only spare phenylalanine in such cases. Until recently a one-step oxidation was postulated for the conversion of phenylalanine to tyrosine. However studies in 1954 by Simmonds *et al.* (52) on a strain of *Escherichia coli* strongly support the view that the conversion is not direct. They suggest that the two acids are synthesized from a common precursor by reversible reactions. It may be that *Paramecium* will be an appropriate form for the future study of this phase of biosynthesis.

Glaucoma and Colpidium.—Kidder, Dewey & Fuller (53) have recently investigated the nitrogen requirements of *Glaucoma scintillans* and *Colpidium campylum*. Both of these ciliates were isolated and established in axenic culture in 1941 (54). The original strain of *Colpidium* has not been maintained. The strain used by Kidder *et al.* was obtained from the culture collection of Cambridge University, England, and was freed of associated bacteria and yeasts by a combined treatment with antibiotics and sulfonamides. In 1942 Peterson (55) was able to grow *Colpidium* in a non-particulate me-

dium containing yeast protein fractions. In the original study, Kidder (54) used a medium containing yeast cell fragments, and because growth failed when the particles were withheld, concluded that they were phagotrophic.

Attempts to culture both of the ciliates in the defined medium for *Tetrahymena* failed (56). The addition of proteose-peptone or casein to the medium did not improve it. However, it was shown later that growth was limited by the level of pantothenate in the *Tetrahymena* medium and when this level was raised tenfold, and casein added, good growth resulted. At the high level of pantothenate neither ciliate grew in the absence of high molecular weight nitrogenous compounds. In the case of *Glaucoma*, the addition of peptone or casein hydrolysate (enzymatic) to a modified *Tetrahymena* medium resulted in good growth, but not up to the level of that induced by whole casein. Casein was subjected to controlled digestion with a number of proteolytic enzymes but even short term action by the enzymes always reduced the activity. The casein was subject to prolonged Soxhlet extraction with 95 per cent ethanol. It was found to support better growth after extraction than before, indicating that the growth-promoting activity may reside in the casein and not in contaminating materials.

Bovine serum albumin (Armour) was substituted for the casein. This protein was utilized readily by *Glaucoma* but not by *Colpidium*. Growth of *Colpidium* was obtained only when casein was present in the medium. Although *Glaucoma* was found to be dependent upon polypeptides, it could utilize free amino acids. The most active amino acid is aspartic acid. Asparagine is inert. With optimum casein concentration (10 mg./ml.), addition of high levels of aspartic acid (2 mg./ml.) increased the growth rate and raised the maximum yield. High levels of glutamic acid produced a similar effect, while the other amino acids did not show marked stimulation individually but increased growth when added together, even in the presence of aspartic acid. Free amino acids were inhibitory to *Colpidium*. The addition of single amino acids or combinations of amino acids to casein-containing media always reduced growth.

These investigators (53) pointed out that Fuller (57), when using low additions of protein which produced low but transplantable growth of *Glaucoma*, was able to show that the omission of any one of the following amino acids resulted in immediate growth failure: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, serine, or proline.

In commenting on their studies on *Glaucoma* and *Colpidium*, Kidder *et al.* suggest that the differences between *Tetrahymena*, *Glaucoma* and *Colpidium* lie in the differences in reactions of the organisms regarding food-taking. All three ciliates are bacteria feeders in nature, where the food organism evokes a swallowing response. They suggest that only *Tetrahymena* has the ability to respond with vacuole formation to relatively small molecules in solution, that the swallowing response of *Glaucoma* is evoked by polypeptides and

small proteins, and that this response is evoked by *Colpidium* only by large proteins.

A requirement for a polypeptide or protein has been reported for other organisms. Sprince & Kupferberg (58) found that the flagellate, *Trichomonas vaginalis*, could be grown on a pancreatic digest of casein to which were added the B-vitamins, purines, a pyrimidine, acetate, ribose, asparagine, linoleic acid, and serum albumin. Growth was dependent on the protein supplement. Dougherty *et al.* (59) found that rhabditoid nematodes, when grown in axenic culture, require a heat-labile protein found in liver and chick embryo juice. Williams & Grady (60) have reported that a specific protein, lactein, is an absolute requirement for the growth of a strain of the bacterium, *Lactobacillus bulgaricus*.

The conclusion of Kidder *et al.* that *Colpidium* requires large protein molecules and that *Glaucoma* requires polypeptides or small proteins, as well as their suggestion that these requirements may reflect the necessity for the stimulation of the swallowing response, is interesting. It must be kept in mind, however, that this conclusion is based on the assumption that the extracted casein and the serum albumen used were pure substances, free from any contaminating materials. It would seem that more work is needed on this point. It is interesting to compare the requirements of *Glaucoma* and *Colpidium* with those of *Paramecium*. Johnson & Miller (39) have shown that *Paramecium* can utilize casein, serum albumin, or a mixture of amino acids, but, in each case, only when small amounts of the nondialyzable fraction of a yeast extract are added. *Paramecium* seems to differ from the other two ciliates in that it requires an additional substance from yeast. Whether this yeast fraction is involved in the swallowing response is not known.

Kidder & Dewey (61) have made a further study of the pantothenic acid requirements of *Tetrahymena*, *Glaucoma* and *Colpidium*. In addition to calcium pantothenate, the following compounds were tested: pantethine, dl-barium-4'-phosphopantetheine, coenzyme A, dl-pantoyllactone and pantothenol. Pantothenol and pantoyllactone were inactive for all three of the ciliates. Calcium pantothenate was 5 to 7 times more active for *Tetrahymena* than was any of the conjugated forms of the vitamin. For *Glaucoma*, on the other hand, dl-barium-4'-phosphopantetheine was the most active form tested, but in all cases the requirements were larger than those of *Tetrahymena*. The most active compound for *Colpidium* was pantothenate. So far they have been unable to find a satisfactory explanation for the high requirements of *Glaucoma* and *Colpidium*.

Carnivorous ciliates and suctorians.—Although the study of the nutrition of carnivorous forms is quite difficult, Lilly and others have made some important contributions in recent years using sterile two-membered cultures. Lilly (62) first established *Stylonychia pustulata* in sterile culture using *Tetrahymena* as the food organism. However, growth was possible only when a supplement was added to the medium. This supplement could be obtained

from yeast and from many plant materials, but apparently not from animal tissues. Another related hypotrich, *Pleurotricha lanceolata*, was found to require the same supplement. Lilly (63) found that growth was proportional to the ciliate food rather than to the concentration of the supplement in both species. He found that none of the known vitamins could be substituted for the supplement.

The growth factor could be concentrated from yeast preparations by adsorption on celite, from which it was eluted by ammoniacal ethanol. In 1952, Lilly & associates (64) obtained a fraction which was active in concentrations of 0.1 $\mu\text{g.}/\text{ml}$. Later Lilly, Sterbenz & Tarantola (65) were able to show that the guanine antagonists, 8-azaguanine, theobromine, theophylline, and caffeine, when substituted for the growth factor, promoted growth. In commenting on these results Lilly concludes that there is such a critical purine balance in *Stylonychia* that the ingestion of a few *Tetrahymena*, with their peculiar guanidine metabolism, is sufficient to prevent growth and that the guanine antagonists apparently affect the balance to the extent that growth on the *Tetrahymena* diet is possible.

While this work was in progress related studies on two suctorians were carried out. Rudzinska (66, 67, 68) has studied the suctorian, *Tokophyra infusionum*, in a bacteria-free culture, using *Tetrahymena* as the food organism. She used autoclaved spring water as the medium. It was found that the feeding of large numbers of *Tetrahymena* shortened the life span of *Tokophyra* and increased the amount of giantism. However, by carefully controlling the number of food organisms, she was able to maintain cultures for several years.

In 1951, Lilly *et al.* (69) found that the addition of yeast extract improved growth and reduced the incidence of giantism and abnormal reproduction in *Tokophyra*. When the yeast extract was omitted, growth did not cease immediately as in the hypotrichs. There was, however, an increase in giantism and in abnormal reproduction and, after several transfers, the cultures usually died out. The growth factor concentrate effective for *Stylonychia* was not effective for *Tokophyra* and it appeared that different chemical entities were involved. Yeast nucleic acid proved to be just as effective as the yeast extract. Even better results were obtained with a mixture of guanylic acid, adenylic acid, cytidylic acid, and uracil. A combination of guanylic acid and uracil was also effective, but individual purines and pyrimidines were without effect (70).

In their 1953 study (65), Lilly *et al.* compared the effect on giantism of increasing concentrations of purines and pyrimidines in *Tokophyra* and another suctorian, *Podophyra collini*, when feeding on *Tetrahymena* in Kidder's medium. In *Tokophyra* there was a favorable effect on growth and reduction of giantism with up to three times the concentration of purines and pyrimidines usually used for *Tetrahymena*, but higher concentrations caused giantism. When they varied the ratio of guanylic acid to uracil in the medium they concluded that the ratio was more important than the concentration,

since the least amount of giantism occurred in *Tokophyra* when the ratio of guanylic acid to uracil in the medium was 13 to 1. They found that the addition of 8-azaguanine reversed this ratio and increased giantism to the maximum. With *Podophyra* the results were just the opposite. Any increase in the concentration of purines and pyrimidines increased the percentage of giantism, while the addition of 8-azaguanine markedly decreased the incidence of giantism. They suggested that the original growth factor may be a natural purine inhibitor.

In commenting on this work, van Wagtendonk (44) states that further work is necessary to clarify the picture. He comments as follows: For example, nothing is known with regard to the content and the ratios of pyrimidines to purines in *Tetrahymena*. These may well have an influence on the interpretation of the data. The addition of inhibitors to two-membered cultures increases the difficulties. These inhibitors have an effect on the food organism as well and might interfere with their metabolism, even to the extent of inhibiting their multiplication. A smaller number of food organisms would then be available for the suctorians, and some of the results obtained by Lilly might be explained in that manner.

Henry, Christy & Lilly (71) have recently studied the nutrition of *Euplotes patella*. When this hypotrich was freed of bacteria, it was found to require several accessory factors when feeding on *Chilomonas paramecium* or *T. pyriformis*. One of these factors was supplied by adding thiamine to the medium. A second factor was supplied either by yeast extract or a mixture of 8-azaguanine and the riboflavin analog, flavotin. The third factor was supplied by an extract of any of five species of bacteria; six other species of bacteria were not adequate sources. This bacterial factor was finally replaced by folic acid. Folic acid and other vitamins of the B complex were ineffective as replacements. As Fauré-Frémiet (72) had described symbiotic bacteria in the cytoplasm of *Euplotes*, tests were made to determine whether the symbionts were present in this study. Feulgen positive particles could not be demonstrated in the cytoplasm of *Euplotes* cultured under bacteria-free conditions.

PHYTOFLAGELLATES

Hutner & Provasoli (73, 74) have provided excellent reviews of the recent work done on this diverse group. The report by Hutner & coworkers in 1949 (75) that *Euglena gracilis* requires vitamin B₁₂ was regarded as a major step forward in the search for a microbiological assay specific for vitamin B₁₂. Since that time a number of investigations have definitely shown the non-specificity of vitamin B₁₂ for *Euglena*. Hendlin (76) found that *E. gracilis* responded to substances of microbial origin other than vitamin B₁₂. Robbins, Hervey & Stebbins (77, 78) have made extensive studies on the vitamin B₁₂ requirements in *Euglena*. These workers isolated over 100 strains of *Euglena* and all required vitamin B₁₂. They have found that pseudovitamin B₁₂ is as active as cyanocobalamin. Cyano-pigment C had about one-half the activity of B₁₂. They also found that a cobalt-containing material isolated by

Elvehjem, which was inactive in the rat, was active for *Euglena*. Mollin & Ross (79) found that urine and plasma samples from both normal and pernicious anemia subjects contained *Euglena*-active substances which were considered not to be vitamin B₁₂.

In 1953 Provasoli & Pinter (80) reported that they had devised chemically defined media for *Peridinium* sp., *Cyanophora paradoxa*, *Synura* sp., and a marine form, *Gyrodinium californicum*. In all cases vitamin B₁₂ was a requirement. More recently Provasoli & McLaughlin (81) have reported that seven species of dinoflagellates have been acquired in bacteria-free cultures. *Amphidinium klebsii* and two unidentified species of *Gyrodinium* were found in sea water. *Amphidinium rhyncocephalum* was isolated from Salton Sea and *Exuviella cassubica*, *Peridinium balticum* and *Peridinium chattoni* were obtained from brackish ponds. All seven organisms require vitamin B₁₂.

Recently, Hutner, Provasoli & Filfus (82) developed a chemically defined medium for the culture of four strains of phagotrophic fresh-water chrysomonads. The organisms studied were *Poteriochromas stipitata* and three different strains of *Ochromonas malhamensis*. They found that the minimal nutritional requirements of these four chrysomonads include vitamin B₁₂, thiamine, biotin (for at least two strains), sugar (or glycerol), and any one of several citric acid cycle components, or related compounds. Involved in this latter requirement are histidine and citric and glutamic acids. Vitamin B₁₂ was spared by methionine but methionine was not spared by choline plus homocystine. The minimal requirements were the same in light and darkness. The authors state that these chrysomonads have the simplest nutritional requirements of any animal hitherto studied, and they recommend their use in studies of the biochemical systems underlying the animal mode of life.

Barber *et al.* (83) have used one of these chrysomonads, *P. stipitata*, as a vitamin B₁₂ assay test organism. In a study involving twenty products and four different methods of vitamin B₁₂ assay, they found that the chrysomonad assay was the only one of the four methods studied that appeared to respond to true vitamin B₁₂ alone. Their results indicated that the chrysomonad does not respond to pseudovitamin B₁₂.

While a considerable understanding of the nutrition and intermediary metabolism of phototrophic euglenids has been obtained in recent years, the animal euglenids have remained almost untouched. Chen (84) first cultivated *Peranema trichophorum* on living *Saccharomyces exiguus* plus soil extract. Following this he obtained thriving cultures on autoclaved cow's milk. In 1953, Storm & Hutner (85) reported that they had been able to determine some of the requirements of this organism. First, they were able to replace milk with cream and water soluble nutrients such as liver or yeast extracts. The cream was replaceable by lecithin and cholesterol. In addition they found that thiamine, riboflavin, vitamin B₁₂ and nucleic acid constituents

(satisfied by uracil, cytidylic, and guanylic and adenylic acids) were essential. In a still later report Hutner & Provasoli (86) state that linoleate could replace most, but not all, of the lecithin. Oleate would not replace linoleate, and in this respect the requirement resembles that found in insects, birds, and mammals. They also found that tryptophan and methionine are required. As traces of fat-soluble and water-soluble unidentified materials are still required, the list of requirements of *Peranema* will ultimately be longer.

Some time ago Lwoff & Dusi (87) had shown that the colorless phytonomad, *Polytomella caeca*, grows abundantly in a salt medium supplying acetate, an ammonium-N source, and thiamine. Recently Wise (88) has investigated a number of substances as possible carbon sources for *P. caeca*. Each tested carbon source was added to a medium containing inorganic salts and thiamine. It was found that each of the following could serve as the sole energy source: propionate, butyrate, valerate; butyl and amyl alcohols, DL-glyceraldehyde, and alpha-ketoglutaric acid. The following compounds would not support growth in the simple medium: caproate, caprylic acid, isobutyrate, or propyl, isopropyl, or isobutyl alcohols. Iso compounds were not utilized. Wise states that so far as his study shows, C₆ seems to be the limit of length of the fatty acids that are suitable carbon and energy sources. *P. caeca* differs from *Polytomella agilis*, studied by Little, Oleson and Williams (89), and *C. paramecium*, studied by Cosgrove & Swanson (90) in utilizing amyl alcohol when grown in simple medium. Both species of *Polytomella* utilize propionate, in contrast to *C. paramecium*. Caproic and caprylic acids, not utilizable by *P. caeca*, support growth of *C. paramecium*.

AMOEBAE

Balamuth & Thompson (91), in commenting on the nutritional studies on rhizopods, point out that studies in this area have lagged well behind those on flagellates and ciliates in spite of the fact that axenic culture of members of all three groups had been attained by the mid-twenties. Oehler (92) in 1924, Cailleau (93) in 1933, Reich (94) in 1933 and Lwoff (95) in 1938 reported exenic cultures of amoebae. Cailleau, Lwoff & Reich had used peptone media in their studies.

In 1951, Storm, Hutner & Cowperthwaite (96) reported that they had been able to replace the crude peptone mixture by a synthetic basal medium supplemented with gelatine hydrolysate and a low concentration of skim milk. In 1953 in a personal communication to Balamuth & Thompson (91), Storm & Hutner gave their medium for the cultivation of *Hartmannella rhysodes* and *Acanthamoeba castellanii*. It contained salts, citrates, acetate, glycerol, glutamic acid, tryptophan, methionine, cystine, asparagine, uracil, cytidylic acid, thiamine, riboflavin and vitamin B₁₂. Their communication indicated that additional amino acids were supplied in the form of gelatin hydrolyzate; that additional B-vitamins were needed but had not been identified; and that an unknown factor, supplied in "vitamin-free" casein

but present in other crude natural materials of both plant and animal origin, was needed. They indicated that tryptophan, methionine, cytidylic acid, thiamin, and vitamin B₁₂ appear to be essential.

Mention of some of the recent work on the nutrition of the ameboid slime molds may not be out of place here. Raper (97) and Raper & Smith (98) reported that *Dictyostelium discoideum* could be grown in two-membered cultures with a wide variety of bacteria and that myxamebae could be cultivated to a limited extent on killed bacteria. In 1952 Bradley & Sussman (99) developed a partially defined medium which permits rapid growth and serial passage for three species. The basal medium consists of yeast extract, beef extract or a purine-pyrimidine mixture, lecithin, a vitamin mixture, salts, and glucose. To this medium must be added a fraction obtained from ground bacterial cells. The fraction is extracted at pH 9.5, is precipitated at pH 7, and is insoluble in organic solvents. It is supplied in the form of an autoclaved paste. Recently Sussman (100) states that this fraction has been isolated in homogeneous form and characterized as a protein of molecular weight between 25,000 and 40,000 containing the usual spectrum of amino acids. The bacterial factor is specific since similar preparations from yeasts or from a variety of plant and animal tissues would not support growth. The purposes served by the bacterial factor have not been completely delineated. The addition of any of a number of proteins to the medium drastically reduces the amount of bacterial factor required but does not eliminate entirely the necessity for its presence.

PARASITIC AND SYMBIOTIC FORMS

Even though many studies have been made on the nutrition of parasitic protozoans, progress has been slow because of their complex requirements. Detailed accounts of this work are to be found in the reviews already mentioned (2, 3, 4). In some of the recent studies progress has been made in the attainment of chemically defined media for a few species.

Trypanosomids and trichomonads.—In 1953 Cowperthwaite, and co-authors (101) reported that they had been able to culture the trypanosomid parasite of mosquitoes, *Herpetomonas culicidarum*, in a completely synthetic medium. This was the first member of the Trypanosomidae to be so cultured. Hemin was found to be required. The essential amino acids included the following: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan, threonine, tyrosine, and valine. These vitamins were required: thiamine, pyridoxamine, riboflavin, pantothenate, nicotinic acid, folic acid, and biotin. In the early phases of the work, a liver fraction was essential for growth. Later it was found that the liver fraction could be eliminated when the concentration of all the vitamins was tripled. The effective vitamins were folic acid and riboflavin, each at high levels sparing the other at still higher levels. Folic acid was greatly spared by the use of certain preparations of adenosine and guanosine, or by low concentrations of crude materials such as certain liver fractions which are virtually devoid of folic

acid. The crude materials were more active on a weight basis than were these ribosides. It was calculated that the folic acid-sparing by crude materials was greater than that permitted by the sum of their riboside and folic acid contents. In the presence of high levels of folic acid, the requirement for exogenous purine was satisfied by any of several purines. In the absence of folic acid the purine requirement was satisfied only by certain commercial sources of adenosine or guanosine. So they concluded that the actual metabolite in natural materials had not yet been identified. In 1955 Nathan & Cowperthwaite (102) indicated that they had partially purified this growth factor for *Herpetomonas* (now called *Crithidia fasciculata*) and named it "Crithidia Factor." They tentatively identified the factor as a polysubstituted pteridine and suggested that it may be the pteridine actually involved in the biosynthesis of functional folic acid.

The failure to propagate hemoflagellates in the absence of erythrocytes or serum has been a major obstacle in analyzing their nutritional requirements. In 1954 Citri & Grossowicz (103) described a liquid medium without blood which supported growth of *Trypanosoma cruzi*. This medium included salts, casein hydrolysate (enzymatic), hematin, bovine albumin, and tomato juice. They were also able to culture *Leishmania tropica* on this medium. More recently they (104) report that they have been able to eliminate the crystalline serum albumin and to substitute known compounds for the tomato juice. Their last medium includes salts, casein hydrolysate, glucose, hematin, Tween 80, and the following substances which replaced the tomato juice: biotin, *p*-aminobenzoic acid, choline, folic acid, inositol, nicotinamide, pyridoxin, pyridoxal, pyridoxamine, riboflavin, thiamine, cobalamin, ribonucleic acid, cytidylic acid, creatine, and creatinine. *T. cruzi* had been carried on the partially defined medium through 27 transfers covering a period of one and a half years at the time of their report. Forty-three strains belonging to the family Trypanosomidae were tested for their ability to grow on the new medium. In addition to *L. tropica* and *T. cruzi*, they obtained growth of strains of *Leishmania infantum*, a strain of *Leishmania* from reptiles, *Trypanosoma acomys*, and *Herpetomonas culicidarum*. The only species tested which did not grow on the medium was a strain of *T. lewisi*. With this start they may ultimately be able to work out a completely defined minimal medium which will lead to a complete definition of the requirements of *T. cruzi* and related forms.

Springe *et al.* (105) have recently reported on their continuation studies on *T. vaginalis*. For growth of this parasite the basal medium must be supplemented with three materials of natural origin: "trypticase" or lipocaic, agar, and human serum. Since lipocaic is an extract from pancreas and as such is likely to be rich in nucleic acids and their derivatives, they investigated the growth stimulation of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), and their derivatives. They found that RNA but not DNA could replace "trypticase" or lipocaic in the medium. The activity of RNA was retained after alkaline or enzymatic hydrolysis, but was destroyed by

acid hydrolysis. None of the hydrolysates of DNA stimulated growth. Sustained growth, equivalent to that with RNA, could be obtained by a combination of the monoribonucleotides or their respective nitrogenous bases, but not by their corresponding ribonucleosides. This was taken to indicate that *T. vaginalis* can convert the free purine and pyrimidine bases, but not their corresponding nucleosides, to the monoribonucleotides essential for growth. They found that at least-adenylic, guanylic and uridylic acids, or their corresponding free bases, are essential for consistently good growth of *T. vaginalis*.

Malarial parasites.—Trager has made some progress in recent years in the extracellular cultivation of the avian malaria parasite, *Plasmodium lophurae*. In 1954 he (106) reported that extracellular development of *P. lophurae* was favored by the addition of coenzyme A to the erythrocyte-extract medium. The effect of CoA was the same regardless of the concentration of the free pantothenate in the medium, indicating that the parasites require the complete coenzyme rather than its pantothenic acid moiety. He found evidence that the parasites are able to accumulate this essential factor which they are unable to synthesize. The CoA concentrations in the livers of infected chickens was approximately 40 per cent lower than that in the livers of control chickens.

In 1955 Trager (107) found that the survival of *P. lophurae* in extracellular culture, for periods up to four days, was favored by the addition of leucovorin. The addition of cocarboxylase or uridine triphosphate had no effect. He also found that in experiments of one or two days no effect could be observed of the omission from the culture medium of added folic acid or p-aminobenzoic acid.

Amoebae.—Balamuth & Thompson (108) have recently reviewed the work on parasitic amoebae. All attempts at axenic cultivation of *Entamoeba histolytica* have failed. Many studies have been made in which one species of associated living bacteria supported growth. In other cases the inclusion of living chicken embryo cells gave growth. Just recently, Bibeau (109) reported *Entamoeba gingivalis* will grow when supplied with living chick embryo cells, as well as when living bacteria are furnished.

In 1953 Phillips (110) described cultures which had been maintained for three years with *T. cruzi* as the associated organism. Some growth was obtained with heat-inactivated *T. cruzi* but no growth was possible with killed *T. cruzi*. He reported that six other species of Trypanosomidae failed to exhibit amoeba-multiplication properties. Last year Balamuth (111) was able to grow the reptilian parasite *Entamoeba invadens* with *T. cruzi* as the associated organism. Balamuth grew *T. cruzi* on a modified egg yolk infusion containing hemin and obtained more abundant growth of the hemoflagellate. Although *E. invadens* grew well in this medium with *T. cruzi*, *E. histolytica* did not. He was able to substitute *Leptomonas oncopelti* which has no hemin requirement, for *T. cruzi* in the culture of *E. invadens*. *E. histolytica*, how-

ever, would not grow when *L. oncopelti* was used, either in the medium devised by Balamuth or in the one devised by Phillips.

Mutualistic intestinal protozoa.—Hungate (112) has recently reviewed the work which has been done on the ciliates of the rumen and on the flagellates of termites and of the roach, *Cryptocercus*. In no instance up to the present has it been possible to culture any of these organisms in an axenic medium. Anyone interested in this field should consult Hungate's review.

CONCLUSION

When the status of our knowledge about protozoan nutrition today is compared with that of five years ago it is apparent that considerable progress has been made. All of the nutritive requirements of *Tetrahymena* are now known. Good progress has been made in determining the requirements of *Paramecium* and some other ciliates. Much more is known about the nutrition of several phytoflagellates. Numerous species have been shown to require vitamin B₁₂. One trypanosomid has been grown in a completely synthetic medium. A good start has been made on the analysis of the requirements of *T. cruzi* and other hemoflagellates, and on at least one free-living amoeba. At least a dent has been made in unraveling the requirements of one species of *Plasmodium*. Axenic cultures of the parasitic amoebae and the mutualistic intestinal protozoa are yet to be attained. With more workers realizing the necessity of working with axenic cultures, our knowledge of protozoan nutrition in the not too distant future should reach such a state that quite significant comparisons of nutritive requirements may be made.

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ANTIBODIES AS INDICATORS FOR BACTERIAL SURFACE STRUCTURES¹

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Antibodies have been used very extensively for the detection, isolation, and identification of the cellular components of bacteria. This work could be applied, however, only in part to an evaluation of the topography of immunologically defined components in the cellular architecture. This correlation has been discussed in detail in two outstanding reviews: in that of Dubos in 1946 (1), and at a Symposium on the nature of the bacterial surface in 1949 (2). The direct visualisation of cellular elements by antibody-reaction was, at that time, restricted to the so called capsular swelling reaction, and to the morphological differentiation of the H, O, and Vi agglutinations. Since then, interpretations of indirect evidence have opened up a fruitful field for speculations, and progress has been achieved in two directions. First the direct visualisation of the effect of antibodies upon certain bacteria has been extended so as to include the cell wall, and secondly, serological reactions have been devised employing certain isolated elements of the bacterial cell. It is the purpose of this review to discuss these recent developments. In doing so, it is realised that the discussion of the observations at present available cannot give a comprehensive picture which would be valid for all groups of bacteria. We feel, however, that these limited observations have opened up a new path to the study of the cellular architecture of bacteria, and justify a review which in part discusses accomplishments, in part possibilities.

DIRECT VISIBLE EFFECTS OF ANTIBODY ACTIONS ON SURFACE COMPONENTS OF THE BACTERIAL CELL

It is generally assumed that antibodies, because of their large molecular size, do not penetrate the cytoplasmic membrane of intact bacteria. No visible effect can therefore be expected inside the cytoplasmic membrane unless this is first destroyed. The permeability of the surface components may be of varying degree; that of the capsule is considerable, that of the cell wall is less so but it can be demonstrated in certain bacteria. There exist no observations demonstrating the permeability of flagella to antibodies.

The visible effects of antibodies upon cells probably parallel the antibody permeabilities of the various surface components; thus, one can detect specific capsular and cell-wall reactions with a phase-contrast microscope. A monomolecular layer of antibody bound on the surface of a single flagellum may elicit an effect observable only in the electron microscope, whereas the bundle of flagella forming a tail may give a visible reaction with antibody

¹ The survey of literature pertaining to this review was concluded in December, 1955, and its preparation was aided by a grant from the Swiss Federal Labour Office (Berne).

which is detectable in dark field or in phase contrast. In this review, the direct visible effects of antibody reactions on bacterial surface structures will be designated as specific capsular, cell-wall, and flagellar reactions.

SPECIFIC CAPSULAR REACTION

This antibody reaction has been known since 1933 as Neufeld's capsular swelling reaction (3). The reasons why this designation must be regarded as erroneous were discussed in detail in a recent review (4). The reaction was discovered by Roger (5) with *Oidium albicans* and was applied by Neufeld (6) to *Diplococcus pneumoniae*. Neither of these authors recognized that the microorganisms they studied were capsulated and thus they described the effect of the antibody as a swelling of the cuticle or as a swelling of the bacterial body. Etinger-Tulczynska (3), who recognized the role of surface components in this reaction, erroneously identified the capsule with the zone of diffraction surrounding the protoplasts which corresponds to the cell wall (4), and she assumed the existence of a second exterior layer, the envelope. Her microphotographs do not show a swelling when India-ink preparations of untreated bacteria are compared with bacteria after their reaction with antibody. The reviewer agrees fully with Duguid (7) who regards the wet India-ink preparation as providing the simplest and most reliable method giving a true picture of the shape and size of the capsule. A number of authors who used this method for comparison were unable to detect any swelling after the addition of anti-capsule serum to microorganisms as varied as *D. pneumoniae*, *Klebsiella*, *Escherichia coli*, *Bacillus anthracis* and *Cryptococcus neoformans* (7 to 11).

A beaded appearance with a regular wave-like margin of the capsular surface was observed after adding the polypeptide antibody to virulent strains of *B. anthracis* when these were grown in inactivated horse serum (12). Do the bulges correspond to an uneven specific swelling of the capsular material on a chain of *B. anthracis*? The comparison of Figures 4 and 5 (Plate I) illustrates that the shapes, the sizes, and the locations of the bulges are the same in the specific capsular reaction as in wet India-ink preparation without the addition of immune serum. The location of these apparent swellings in the area of division of the bacilli indicates that the areas of production of capsular material are probably concentrated in the proximity of the cross walls.

In those studies in which a relative capsular swelling following application of antibodies was observed in the electron microscope (13), or by using a quantitative centrifugal method (14), the volume increase was small. Johnson & Dennison (14), cautiously stated that the volume increase appeared to be somewhat greater than the aggregate volume of the antibody molecules adhering to the capsule. According to them the reaction is fundamentally one of precipitation. Until we know more about the nature of this reaction, it may be more appropriate to speak about changes of refraction following the specific binding of antibody molecules which have diffused into the capsular material to varying distances rather than about swelling. On the

basis of direct microscopic observation the depth of such diffusion must be more than that calculated by Hershey (15).

The author is aware that it is not easy to abandon expressions as generally used as "capsule swelling" or "the Neufeld effect" (16), but on the other hand it does not seem logical to speak about a swelling reaction when this occurs only occasionally as a secondary hydration effect. While investigating a nonspecific pH dependent capsular reaction brought about by proteins, Tomcsik & Guex-Holzer (17) found that sometimes a secondary swelling, sometimes a secondary shrinkage can be observed, depending upon the pH. It would be, indeed, absurd to designate a reaction leading to a secondary dehydration as a swelling reaction.

Our conception of the nature of the specific capsular reaction permits the use of antibodies as microanalytical tools for the topographical detection of different capsular substances. Before discussing this subject, however, we should examine the nature of the evidence which allows conclusions regarding the capsular derivation of substances isolated by extraction of the bacterial cell. In the case of *Klebsiella pneumoniae* it was relatively easy to assume the capsular localisation of the polysaccharide first isolated by Toennissen (18) from encapsulated strains by extraction with hot KOH, since no such substance could be obtained by applying the same procedure to noncapsulated forms. Similar conclusions could be drawn from the classical work of Heidelberger and Avery concerned with the soluble specific substances of capsulated strains of *D. pneumoniae*. Their conclusions were definitely corroborated cytologically, since only the polysaccharide specific substance was able to absorb the antibody which gave the specific capsular reaction.

It was more difficult to interpret the derivation of the material obtained by Kramár (19) by alkali-extraction of capsulated *B. anthracis*, and designated by him as a glycoprotein. The serological activity of this material was not examined. The lesser known arguments (1) of our early work (8, 20 to 23), which led to the discovery of D-glutamic acid polypeptide should be mentioned briefly inasmuch as they elucidate the derivation of the extracted material. A polysaccharide was first isolated, and this was found to have the same serological activity and to occur in approximately the same quantity, regardless of whether the extraction was carried out on capsulated or non-capsulated bacteria. In consequence, the probability that this polysaccharide corresponded to the capsular substance was small. A protein-like material, designated as P substance, was obtained only by extracting capsulated *B. anthracis*. This substance could be separated from the specific polysaccharide by CuSO_4 precipitation, and its totally different serological specificity was established. The P-antibody agglutinated capsulated anthrax bacilli, and rendered the capsule visible (specific capsular reaction), whereas the polysaccharide antibody did not produce these reactions. The definite conclusion could thus be drawn, that the P substance derives from the capsule, whereas the polysaccharide occurs in the bacterial body. The topographical localisa-

tion of the polysaccharide in the cell wall was cleared up much later with the aid of the specific cell-wall reaction (12). The D-glutamic acid polypeptide nature of the P substance was discovered by Ivanovics & Bruckner (24).

Somewhat similar difficulties arose over the nature of the capsular substance of *Pasteurella pestis*. A serological difference between the surface of capsulated and noncapsulated plague bacilli was noted by Schütze (25). He regarded the slimy hydrated surface-layer of these bacteria as envelope since it was readily dissolved by heating the bacterial suspension to 60°C. Baker *et al.* (26, 27) isolated from the "envelope solution" a carbohydrate-protein complex and a carbohydrate-free protein. Both fractions seemed to be identical immunologically. Amies (28) confirmed these observations, but he could obtain only the carbohydrate-free protein from the surface structure, which he regarded as a capsule. Englesberg & Levy (29) listed several arguments for the identity of both the protein and of the carbohydrate-protein complex fractions with the envelope. The most important of them seems to us to be that an immune serum deprived of protein antibody by specific absorption with these combined fractions "fails to form aggregates on the envelope." Certain evidence speaks thus for the occurrence of two substances in the capsule of *P. pestis*, although a topographical difference could not be demonstrated and the cellular localisation as well as the immunological significance of the polysaccharide was not clearly defined.

One of the main limitations in using the specific capsular reaction for the detection of different capsular substances is that free polysaccharides and their complexes may give an identical reaction with antiserum. The numerous observations which show that intact, capsulated microorganisms produce antibodies in rabbits, whereas the isolated polysaccharides and polypeptides do not, suggest either that these substances are different in the native state, or that they occur in the capsule at least partly in the form of complexes. Dubos (1) suggested a ribonucleic acid combination in *D. pneumoniae*, whereas Taylor (30) suggested a dual structure for the capsule, consisting of an external hapten layer and an internal layer in which the same material forms a complex with an unknown substance.

More concrete immunochemical evidence can be collected to illustrate the complex chemical structure of the capsule in *Streptococcus haemolyticus* and in *Haemophilus influenzae*. According to Lancefield (31), the M substance of *Streptococcus* can be removed by proteolytic digestion without affecting the viability of the cell. If this substance is regarded as a protein lying on the external surface of the cell wall, one could assume that it corresponds actually to the internal layer of the capsule, the exterior being formed by the hyaluronic acid layer visible in young cultures. Williamson & Zinnemann (16, 32) reported that six freshly isolated *H. influenzae* strains reacted only with some of the e-type immune sera. After partial degradation of the capsule, however, these bacteria reacted with all e-type immune sera. Cross "Neufeld reactions" suggested the presence of two type specific e antigens. One of these, e 1, is more superficial and occurs only in freshly iso-

lated strains. The e 2 antigen is situated deeper in these strains but it is the only capsular antigen present in old cultures. The complex capsular structure of *Streptococcus* and of *H. influenzae* can thus be assumed on the basis of indirect evidence. No direct visible specific capsular reaction has been reported as yet giving information on the superimposition of the two supposed layers.

A complex capsular structure visible after the addition of polysaccharide and of polypeptide antibody was discovered by Tomcsik in *Bacillus megaterium* (33). The individual effects of these antibodies for some species of the genus *Bacillus* had been previously demonstrated. Bodon & Tomcsik (8) found a specific capsular reaction with polypeptide antibody on *B. anthracis*. Ivanovics & Erdös (34) showed the presence of polypeptide in the capsule of bacilli belonging to the subtilis group as well as of bacilli similar to *B. megaterium*. Aubert & Millet (35) extracted a polysaccharide by boiling an aqueous suspension of capsulated *B. megaterium* for two hours. They assumed the capsular derivation of this polysaccharide on the basis that the capsule of boiled bacteria could not be stained with Giemsa, in contrast to that of nontreated bacteria. This is meager evidence, indeed, in view of the criteria set up in the discussion of the capsular substance of *B. anthracis*.

Tomcsik & Guex-Holzer (33, 36) found in their study of ten well defined species of the genus *Bacillus* that only *B. megaterium* gave a complex capsular reaction when both anti-anthrax-polypeptide and a homologous antibody were applied to the capsulated bacteria. The antibody reacting with D-glutamic acid polypeptide could only be produced in rabbits with capsulated anthrax bacilli in sufficient concentration using a procedure first described by Tomcsik & Szongott (21). The capsular polypeptide detected with this antibody appeared in homogeneous distribution invariably in *B. anthracis* and in *B. subtilis* and usually in *B. megaterium*. The species or type specific homologous antibodies did not give any reaction with the capsules of *B. anthracis*, or of *Bacillus subtilis* but they revealed a surprising type specific capsular structure on *B. megaterium*. The most regular appearance of this capsular structure could be observed in several hundreds of subcultures of a variety of *B. megaterium* which was designated as *Bacillus M*. The essential elements of the capsular structure made visible with the homologous antibody were transverse septa separating the individual bacilli within the chain and disc-like condensations at both poles of the chain (Plate I, Fig. 2). Furthermore a marginal as well as a transverse striated reaction appeared in varying degree. An immunochemical analysis (36, 37, 38) showed that the antibody revealing these structural elements of the capsule reacts specifically with a mucopolysaccharide which also occurs in the cell wall. It seemed justifiable to conclude therefore, that the capsule of *B. megaterium* is a complex structure, consisting of a polysaccharide framework, the spaces being filled out with the polypeptide. Since the polypeptide is present in considerably larger quantity than the polysaccharide, and the polysaccharide structures are apparently thin, the specific capsular reaction of *B. megaterium*

most usually reveals a homogeneous appearance when examined with polypeptide antibody alone (Plate I, Fig. 1).

At this stage of the work the conclusions of Tomcsik and Guex-Holzer were contested by Ivanovics and Horvath (39, 40), who claimed that the framework consists of polypeptide, and that the polysaccharide is given up mainly as a slime. We are not able to understand their interpretation for the following reason: they also found by using the polypeptide antibody, that the distribution of the capsular material appeared most frequently to be homogeneous. In a certain percentage of their observations, the polypeptide antibody revealed a narrow striation, but this was totally dissimilar to the absolutely regular locations of the polysaccharide transverse septa.

We think that the apparent controversy was fully cleared up in our later work. In order to collect information on the derivation of the polysaccharide transverse septa, the capsulated form of *Bacillus M.* was subjected to digestion by lysozyme. This experiment led to the first observation regarding the spherical transformation of the bacterial protoplasts following lysozyme action (41). In the initial stage of this action, the separation of the protoplast-surface from the cell wall was brought about by dilatation of the cell wall. The permeability of the capsule and of the cell wall to the polysaccharide antibody increased, and the transverse septa were revealed as forming direct continuations of the cross cell walls.

Further information regarding the derivation of the complex capsular structure has been obtained through observations of the initial stages of capsule formation (42). A noncapsulated form of *Bacillus M.* can be obtained in aerated bouillon culture. By transferring this organism to agar medium, the initial phases of the capsule formation have been studied by using both polypeptide and polysaccharide antibody. The polypeptide appeared on the surface of the cell wall in 1 to 2 hr. in the form of irregularly located patches except at the division-level where, adjoining the periphery of the cross wall, a knob-like polysaccharide mass appeared giving a bamboo-like appearance to the chain. At a somewhat later stage the knob-like structures were pushed outwards following the further production of polysaccharide material (Plate I, Fig. 3) until in the fully developed capsules they were no longer perceptible. The complementary nature of the location of polysaccharide structure and of polypeptide material is clearly visible in the exceptionally well developed capsules shown in Plate I, Figures 6 and 7.

During the course of five years of observations, the complex capsular structure of *Bacillus M.* has not shown any essential modifications in spite of frequent transfers on agar medium. Certain variations were, however, observed in the recent work of Tomcsik & Baumann-Grace (43) in a study of a larger number of *B. megaterium* strains. A considerable portion showed the typical complex capsular structure when freshly isolated from the soil. Some of them (e.g. Mg 5) produced, however, an anomalous capsular structure. The polypeptide formed the exterior, the polysaccharide the interior layer of the capsule, and polysaccharide cross septa did not project to the surface of

the capsule (Plate I, Figs. 8 and 9). A similar circular arrangement of the polypeptide and polysaccharide capsular layers was also observed in an old laboratory culture of *B. megaterium* (Mg 2); in this case there were no capsular transverse septa (Plate II, Figs. 10, 11, and 12).

Fading effect.—Occasionally, extremely compact polypeptide capsules were observed (43) which could not be dissolved by boiling the bacterial suspension for 10 min. in *N*/10 HCl or in *N*/10 KOH. The protoplasts of these bacteria could be digested with trypsin, but the polypeptide still adhered tightly to the empty cytoskeleton. By adding normal serum to such bacteria, the capsule became discernible by a change of refraction, whereas the visibility of the cell wall was considerably reduced, giving the impression of a fading effect. The polysaccharide antibody behaved in a similar way with respect to capsulated chains, but it elicited a specific cell-wall reaction on the noncapsulated chains deprived of polypeptide (Plate II, Fig. 13). The polypeptide antibody reacted in a normal way with the capsulated chains (Plate II, Fig. 14).

Pseudoreaction.—The specific capsular reactions are detectable in phase contrast usually within 1 min. after the addition of antibody. The reaction on living bacteria is less regular when the preparation is held for 24 hr. in a wet chamber probably because of the beginning of autolysis. In studying the effects of different antibodies on the capsule of *B. anthracis*, the reaction of polypeptide antibody was seen to appear within a few seconds, whereas no reaction was observed until 17 hr. after the addition of polysaccharide antibody (12). At this time thin irregularly located "cross septa" appeared in the capsule (Plate III, Fig. 21). After further incubation, the "septa" became thicker and in 30 hr. globular condensations appeared at their distal ends (Plate III, Fig. 22). This reaction could be elicited only with sera containing antibody to the anthrax polysaccharide. The antibody reacts primarily with the cell wall but prolonged incubation probably leads to the extrusion of protoplasmic material; thus a capsular pseudoreaction arises during the long incubation in the absence of preexisting structures.

SPECIFIC CELL-WALL REACTION

The permeability of the capsule to antibodies may be limited and they may not be able to diffuse through the whole breadth of the capsule. This can be seen, for example, in the polysaccharide transverse septa of *B. megaterium* which are frequently rendered visible only in their distal portions (41). The polypeptide antibody, if present in sufficient quantity, diffuses usually up to the surface of the cell wall, which then appears as a diffraction zone between the bacterial body and the capsule (4). If polysaccharide and polypeptide are simultaneously present, the diffraction zone may disappear. When cell wall antibody reacts with noncapsulated bacteria, a visible change can thus be expected; this is, however, less noticeable in phase-contrast than in the electron microscope.

A visible change in the cell wall brought about by immune serum to intact

bacteria was discovered by Mudd & Anderson (44). They demonstrated with the electron microscope that the transparent cell walls of *Salmonella typhosa* and *B. subtilis* assume an optical density similar to that of the cytoplasm when the homologous "somatic" antibodies are added. An analogous apparent thickening of the bacterial bodies may or may not be visible in phase-contrast. Recent work has demonstrated, however, that in several bacteria a visible antibody reaction occurs on the cell wall if this is separated from the cytoplasm by enzymatic or mechanical means.

An enzymatic separation of the cell wall from the cytoplasmic membrane and cytoplasm can be most ideally accomplished by the lysozyme digestion of *B. megaterium*, as Tomcsik & Guex-Holzer first demonstrated (41). In the initial stage of lysozyme action the cell wall, owing to the partial depolymerisation of its mucopolysaccharides, loses its elasticity and becomes separated from the cytoplasmic membrane. The forces leading to the progressive dilatation of the cell wall and thus permitting the spherical transformation of the intact protoplasts, are unknown. At this initial stage the cell wall is hardly visible in phase contrast (Plate II, Fig. 15) but it can be seen, when

EXPLANATION OF PLATES



Photographs 1 to 7 and 21 to 29 previously published (12, 42, 47, 106). Photographs 8 to 20 not previously published (43). Magnification $\times 2500$. Phase-contrast: Wild & Co., Switzerland.

LEGENDS

Plate I

FIG. 1. Capsulated *Bacillus M.*; polypeptide antibody.

FIG. 2. The same microscopic field as Fig. 1; subsequent addition of polysaccharide antibody. Capsular transverse septa and polar concentration of polysaccharide material.

FIG. 3. Noncapsulated *Bacillus M.*, from aerated bouillon culture, transferred to agar. After 5 hr. at 28° C. secondary capsule production. The polysaccharide antibody renders the marginal concentration of the transverse septa visible.

FIG. 4. *Bacillus anthracis* "Vollum" grown 24 hr. in inactivated horse serum. Wet India-ink preparation.

FIG. 5. The same culture as in Fig. 4; polypeptide antibody. The beaded appearance of the capsule is attributable to the more intensive polypeptide production at the division level. No capsular swelling.

FIG. 6. Originally noncapsulated *Bacillus M.* from aerated Sauton culture; secondary capsule production in the centrifuged sediment at room temperature in 3 days. Polysaccharide antibody. Note the unusually thick transverse septa.

FIG. 7. Bacteria as in Fig. 6. Polypeptide antibody. The distribution of the secondarily produced polypeptide is not homogenous. Gaps in place of the polysaccharide transverse septa.

FIG. 8. *B. megaterium* (Mg 5) freshly isolated from soil. Margin of the broad capsule depicted with diluted polypeptide antibody. The homologous polysaccharide antibody reacts with the interior part of the capsule and reveals a striated material with capsule septa.

FIG. 9. The same bacterium after the subsequent addition of undiluted polypeptide antibody.



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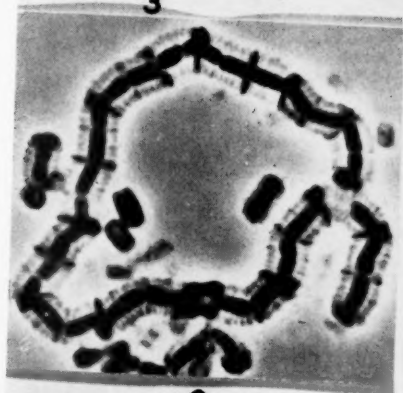
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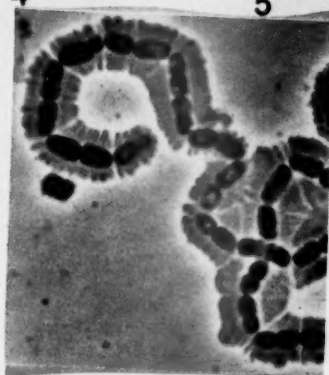
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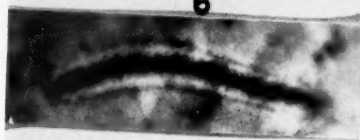
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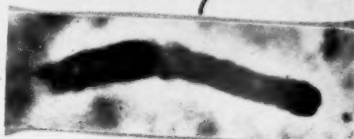
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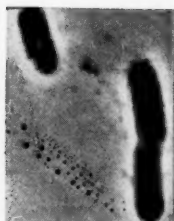
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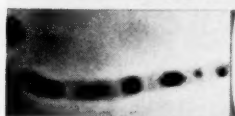
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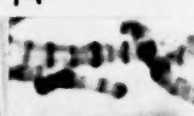
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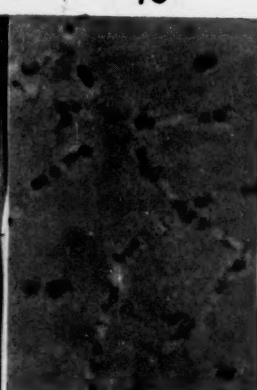
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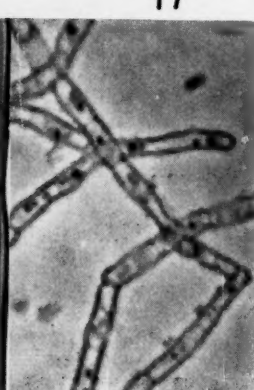
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the elements are moving with the current, that the rows of ellipsoidal or spherical protoplasts are still being held together. The addition of the homologous polysaccharide (but not of the polypeptide) antibody renders the cell wall and the numerous cross walls distinctly visible (Plate II, Figs 16 and 17; Plate III, Fig. 26). We recognized the similarity between this and the specific capsular reaction and we designated it, therefore, as the specific cell-wall reaction. With the help of this reaction the subsequent morphological stages of the cell-wall disintegration could be followed up to the total dissolution of the cell wall. Since many of the fragile protoplasts of *Bacillus M.* were still intact at this stage of the lysozyme action, even when the suspending fluid was 0.85% NaCl, it could be concluded that the lysozyme acts primarily on the cell wall and not on the protoplasts. The subsequent observation of Weibull (45), that the protoplasts may be preserved for several days when this reaction is carried out in 0.1 to 0.2 *M* sucrose solution, strengthened this conclusion. At the same time Salton (46) observed that the isolated cell walls of *Micrococcus lysodeikticus* are dissolved by lysozyme. This observation was to be expected in view of Fleming's discovery that these organisms undergo total dissolution. The point is that lysozyme acts primarily on the cell wall, and the dissolution of the protoplasts is a secondary phenomenon depending partly on the varying resistance of the *B. megaterium* protoplasts, partly on the suspending fluid. The specific cell-wall



Plate II

FIG. 10. *B. megaterium* (Mg 2). The homologous polysaccharide antibody reacts with the interior part of the capsule.

FIG. 11. *B. megaterium* (Mg 2). The polypeptide antibody reacts with the surface layer of the capsule.

FIG. 12. *B. megaterium* (Mg 2). Polysaccharide as well as polypeptide antibody added. The interior layer consists of polysaccharide, the exterior one of polypeptide.

FIG. 13. *B. megaterium* (Mg 5) treated with trypsin. The cytoplasm was digested, leaving the cell wall and the capsule intact. The added polysaccharide antibody reacts with the noncapsulated bacteria but causes a "fading" effect on the capsulated ones, probably as a result of the unusually compact polypeptide capsule.

FIG. 14. The same microscopic field as in Fig. 13 after the successive addition of polypeptide antibody.

FIG. 15. *B. megaterium* (Mg 1) suspended in 0.2 *M* saccharose solution containing 1:400 egg-white. 15 min. room temp. Spherical transformation of protoplasts, cell wall slightly visible.

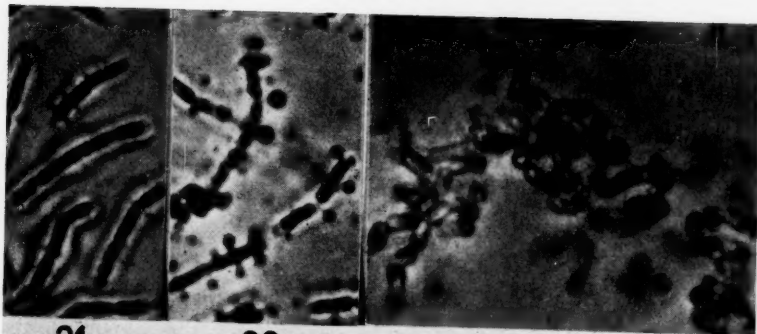
FIG. 16. The same suspension as in Fig. 15 after the addition of homologous polysaccharide antibody. Specific cell-wall reaction with fragmentation of the chain.

FIG. 17. *Bacillus M.* four-days-old autolyzed culture. Polysaccharide antibody. Strong reaction of the transverse septa.

FIG. 18. *B. megaterium* (Mg 1) treated with trypsin. The cell wall is faintly visible without antibody.

FIG. 19. The same microscopic field as in Fig. 18 after the addition of normal serum. "Fading" effect.

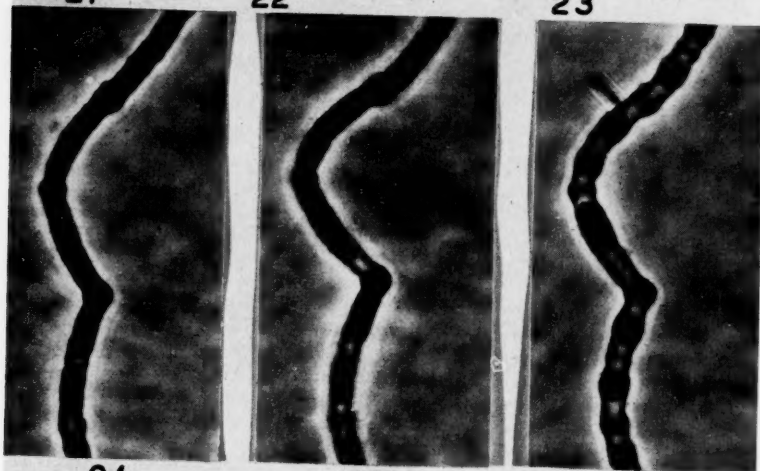
FIG. 20. The same microscopic field as in Fig. 19 after the consecutive addition of homologous antibody. The "fading" disappeared. The specific cell-wall reaction is very marked.



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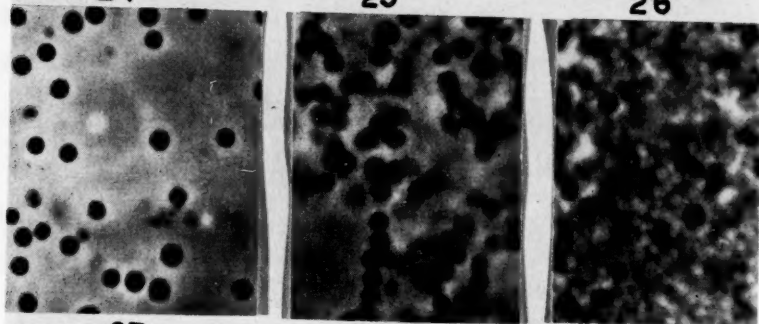
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reaction was observed in capsulated as well as noncapsulated *Bacillus M.* after appropriate lysozyme treatment (41, 42, 47). The capsular polypeptide went into solution as the polysaccharide capsular septa disintegrated; both disappeared during the initial phases of the cell-wall dilatation. It could thus be assumed that the lysozyme substrate occurs in the capsular framework as well as in the cell wall and in the cross wall.

Meyer *et al.* (48, 49), Epstein & Chain (50), Feiner *et al.* (51), and Webb (52) found that reducing substances are set free when lysozyme acts upon sensitive bacteria or on the mucopolysaccharides isolated from them. The amount of these substances was at most one-third that obtained by acid hydrolysis. The lysozyme-treated mucopolysaccharide was still active serologically. This observation of Feiner *et al.* (51) led Guex-Holzer & Tomcsik (38) to isolate the polysaccharide of the cell wall from *Bacillus M.* by lysozyme treatment of the cells. Enzymatic extraction had to be considered in preference to the more conventional methods of extraction using alkalis, acids, sodium taurocholate, phenol, formamide and antiformin because these latter did not lead to the extraction of serologically highly active material. The lysozyme extraction was carried out cautiously, and immediately stopped when the cell wall disappeared, using the specific cell-wall reaction as an indicator. From the chilled extract a mucoprotein was isolated having the following composition: N 7.6 per cent; P 4.7 per cent; reducing substances after acid hydrolysis 18 per cent; acetyl 4 per cent. Galactosamine, glucosamine, diaminopimelic acid, alanine, glutamic acid, and two other unknown ninhydrin-reacting substances were disclosed in the hydrolysate



Plate III

FIG. 21. *B. anthracis* "Vollum" grown 24 hr. in 1 per cent glucose containing inactivated horse serum. Anthrax polysaccharide antibody added to the wet preparation, which was kept afterwards in wet chamber. No visible reaction within 3 hr. After 17 hr. at room temperature thin transverse lines appear in the capsule.

FIG. 22. The same preparation as in Fig. 21 after 30 hr. in wet chamber. Pseudo-reaction, following long incubation.

FIG. 23. *Bacillus M.* from aerated bouillon culture treated with trypsin. The addition of homologous polysaccharide antibody causes cell-wall agglutination.

FIG. 24. *Bacillus M.* R-type suspended in 0.2 *M* sucrose solution containing 1:400 egg white. Homologous polysaccharide antibody added to the wet preparation. Photographed 10 min. after the addition of egg white.

FIG. 25. The same microscopic field as in Fig. 24, 5 min. later. Specific cell-wall reaction and initial stages of spherical transformation of the protoplasts.

FIG. 26. The same microscopic field as Fig. 25, 45 min. later. The spherical transformation is completed.

FIG. 27. *Bacillus M.* Free protoplasts obtained through complete lysozyme digestion of the cell wall in 0.2 *M* sucrose solution.

FIG. 28. Agglutination of fresh protoplasts of *Bacillus M.* with protoplast antibody.

FIG. 29. Agglutination of autolyzed protoplasts (cytoplasmic membrane) of *Bacillus M.* with protoplast antibody.

by chromatographic analysis. A similar mucoprotein could be obtained by the lysozyme extraction of both noncapsulated and capsulated *Bacillus M.*, although the extraction of the latter was stopped immediately after the dissolution of the capsule, when the cell walls were still fairly intact and still gave the specific cell-wall reaction with polysaccharide antibody. The term "polysaccharide antibody" was also used further since the same antibody reacts with a typical mucopolysaccharide isolated with hot water from capsulated microorganisms (38). The mucoprotein apparently is a less degraded form of an unknown, probably more complex substance which occurs in the intact capsule or in the cell wall. Both lysozyme-extracted mucoproteins gave a precipitation reaction with homologous serum when they were diluted up to 1:10⁶, and both absorbed from immune serum the antibody responsible for the specific cell-wall reaction as well as that which made the characteristic capsular framework visible. We believe that the identity of composition of the cell wall and of the capsular framework revealed by the specific capsular and specific cell-wall reactions carried out with defined antibodies could not have been demonstrated by any other method known up to now.

The cytochemical application of certain color reactions lacks the appropriate specificity and in certain cases even the sensitivity of the serological method. The periodate-Schiff polysaccharide stain of Hotchkiss (53) and McManus (54), applied by Pennington (55) to the cytochemical investigation of microbial structure was not tried, but the Alcian blue method, recommended by Novelli (56) and McKinney (57) for staining capsular polysaccharides, did not reveal the framework (58). It is difficult to explain this negative result since Tomcsik & Grace (58) obtained good results with Alcian blue in the selective staining of the cell wall in *Bacillus M.* as well as in a number of other microorganisms.

Since the enzymic separation of the cell wall and of protoplasts can be carried out so far only with a few microorganisms, the specific cell-wall reaction was further studied using cytoskeletons obtained by the digestion of protoplasts with proteolytic enzymes. Contrary to the observation of Kantorowicz (59), Salton & Horne (60), and Salton (61), according to which Gram-positive microorganisms are not amenable to the action of proteolytic enzymes, Tomcsik & Guex-Holzer (47) found that the cytoplasm of *Bacillus M.* can be digested with trypsin, pepsin, and papain, and its cell walls still give a visible cell-wall reaction (Plate II, Figs. 18 to 20). This observation has been extended by Tomcsik & Grace (58) to other types of *B. megaterium* and to some strains of *B. anthracis*. Even certain strains of *B. subtilis* seem to be amenable to trypsin digestion according to our recent work (43) and to a microphotograph we found in a recent paper of Yoshida *et al.* (62). The behavior of the bacteria toward proteolytic enzymes certainly requires a reinvestigation before definite recommendations can be made regarding the use of this method for the study of the specific cell-wall reaction. Occasionally even autolytic enzymes may leave an intact cytoskeleton which reacts with antibody (Plate II, Fig. 17).

A method which is of more general use in the study of the specific cell-wall reaction consists of the removal of cytoplasm following the mechanical disruption of the cell wall. This can be accomplished very simply by crushing the bacterial preparations with coverslip forceps as shown by Murray & Robinow (63) in their studies on the disposition of the cell wall of *Bacillus cereus*. More uniform results can be attained, however, by powerful mechanical agitation. Mudd *et al.* (64) used the sonic oscillator for this purpose with good effect. After the demonstration that minute glass beads considerably enhance the effect of agitation (65, 66), Dawson (67), and Salton & Horne (68) obtained excellent results by shaking thick bacterial suspensions with these glass beads in the Mickle disintegrator (69). The cell walls were split, the cytoplasmic membranes destroyed, and the cytoplasm went into solution. Salton (91, 92, 93) used the centrifuged sediment for the chemical analysis of the cell wall. Tomcsik & Grace (58) observed that cell walls prepared in this way reveal the characteristic structure of multicellular bacteria demonstrated previously by classical staining methods. Apart from several species in the genus *Bacillus*, the septate nature of *Caryophanon latum* (70) and of certain *Micrococci* (71) was confirmed. Similar preparations gave a distinct specific cell-wall reaction in *B. megaterium*, in *B. cereus*, and in *B. anthracis*. The reactions observed by applying homologous agglutinating immune sera to cell-wall preparates of *C. latum* and to septate *Micrococci* was less distinct since the cell walls and the cross walls of these microorganisms were thick and clearly visible structures even before the addition of antibody. A secondary swelling revealing a maltese cross-like form was found in the septate *Micrococci* and thus, in these bacteria, some visible effect of the antibody reaction could still be elicited. On the basis of these limited observations it is questionable whether the specific cell-wall reaction would be visible in the majority of bacteria in phase contrast. Perhaps the development of the preparative technique for the electron microscope will permit a more general application of this reaction. Such an assumption is encouraged by the recent observation of Yoshida *et al.* (72) with *S. typhi* cell walls obtained by mechanical agitation. The cell walls were washed carefully after their contact with homologous antibody. After subsequent treatment with phosphotungstic acid solution an increased contrast was noted in electronmicrographs.

The technique of the specific cell-wall reaction in phase-contrast is naturally much simpler, and the interpretation of observations is much easier, than in the case of electron microscopic studies. In the most distinct observations made with *Bacillus M.*, a dumb-bell like form of the cross cell wall and a knob-like protuberance at the surface of the cell wall at division level (42) can be demonstrated; these have not been observed previously by classical cytological methods. These may well be the products of the "growing points" postulated by Bisset (73, 74, 75) in his lucid studies on the mechanism of cell-division. The main advantage in the use of the specific cell-wall reaction is that, with its help, the occurrence of immunochemically defined substances can be detected in the cell wall.

SPECIFIC FLAGELLAR REACTION

Using dark ground with especially strong illumination, a visible change on the surface of flagella or on the surface of tails composed of bundles of flagella was discovered by Pijper (76, 77, 78) after the addition of homologous H antibody. The motility of *S. typhosa* after the addition of antibody becomes first irregular, the bacillus turning about in a jerky spasmodic manner, then it is abolished. The tails become covered with granules of various sizes which may coalesce to form a complete sheath transforming the tail into a stiff, thick spiral. We designate this change, which under appropriate circumstances is visible by ordinary microscopy, as the specific flagellar reaction since it is in principle similar to the specific capsular and to the specific cell-wall reaction. Depending on the currents in the suspending fluid, the specific flagellar reaction leads first to contact then to attachments of the tails of different cells resulting in the formation of the characteristic loose H agglutinates.

The specific flagellar reaction was studied in the electron microscope by Mudd & Anderson (44), who observed a thickening of the individual flagella on *S. typhosa* and on *B. subtilis* after the addition of the homologous antibodies. The thickening attributable to the specific flagellar reaction could be measured, and it corresponded to a film of radially disposed globulin molecules.

In his later work (79, 80, 81), Pijper regarded the flagella as an appendix of the cell wall or of the capsule, whereas on the basis of electron microscopic studies (82 to 86) a cytoplasmic origin has been unanimously assumed.

In a few cases flagella have been studied in the isolated state and an essential difference has been found between their chemical composition and that of the capsule and the cell wall. In their fundamental studies Weibull (87, 88, 89) and Astbury (90) discovered the chemical nature of the flagellar material of *Proteus vulgaris* and *B. subtilis*; in their chemical composition these entities are included in the great family of elastic fibrous proteins. The flagella contain a large number of amino-acids, but they lack cysteine, tryptophan, histidine, and hydroxyproline. Serological studies of Weibull (88) showed that the isolated flagella of *P. vulgaris* produced antibodies in rabbits which agglutinated the corresponding bacterial suspension to as high a titer as an antiserum produced by the bacterial suspension itself.

SEROLOGICAL REACTIONS OF ISOLATED CELL COMPONENTS

SEROLOGICAL REACTIONS OF THE CELL WALL

Apart from the specific cell-wall reaction which elicits a microscopically visible effect of antibody on the cell wall, another approach has been recently introduced in the study of the topography of surface antigens of bacteria, using the simple methods developed for the separation of the bacterial cell wall from the cytoplasm as discussed in the section on the specific cell-wall reaction.

One of these methods was used by Salton (68, 91, 92, 93) in his fundamental studies on the chemical composition of the bacterial cell wall. He prepared the cell walls by shaking the bacterial suspension with minute glass beads in the Mickle disintegrator (68). The cytoplasm of more than 90 per cent of the bacteria can be brought into solution by this procedure, and the empty cell walls may be isolated by differential centrifugation. In our opinion, however, it is not certain whether the cytoplasmic membrane still adheres in part to the interior surface of the cell wall, thus slightly influencing the result of the chemical analysis. This possibility seems likely since it is known that, in many species of bacteria, the cytoplasmic membrane is not easily separated from the cell wall following plasmolysis. Unfortunately no microscopic method is as yet known which might determine whether this is so, although serological analysis might give some useful information regarding this question.

The cell walls of *Streptococcus faecalis* prepared by Salton (91) were insoluble in Schweizer reagent, formamide, diethyleneglycol, trichloroacetic acid, pyridine, or phenol. They gave a strongly positive Molisch and a weakly positive Biuret reaction. Following acid hydrolysis the polysaccharide content was estimated as 60 per cent and the protein content, calculated on the basis of 5.1 to 5.4 per cent nitrogen, as 25 per cent. Lipoid material was present in less than 2 per cent. In subsequent works, the analysis of cell walls has been extended to *Streptococcus pyogenes*, *M. lysodeikticus*, *Sarcina lutea*, *B. subtilis*, *E. coli*, and *Salmonella pullorum* (92, 93). The cell wall of gram-positive bacteria contained more hexosamine and less lipoid, and a smaller number of amino-acids when compared to those of the gram-negative microorganisms. With the exception of *S. pyogenes*, Salton did not apply immunochemical methods to the characterisation of his cell-wall preparations. Since the two reducing substances, rhamnose and hexosamine, found in the streptococcus cell-wall hydrolysate, were identical to those reported previously (94) in the carefully purified streptococcal group A substance, Salton posed the question whether the latter substance is actually a component of the rigid cell wall. By extracting the *S. pyogenes* cell wall by the classical methods employed to obtain group A substance, he obtained an affirmative answer to this question. The same result was reported somewhat earlier by McCarty (95) who used Maxted's method (96) to extract the streptococcus cell walls for the isolation of group A substance. The cell walls were dissolved by the same fractions of the *Streptomyces albus* enzymes that were effective in the lysis of intact cells; this permitted the conclusion that the carbohydrate component of the cell wall, which is composed primarily of N-acetyl-glucosamine and rhamnose, is the group specific C carbohydrate.

The majority of previous immunochemical studies gave only indirect evidence of the kind necessary to establish the topographical location of cellular antigens. It is generally assumed that in the agglutination reaction only those antigens which are freely exposed at the surface of the cell are involved. With this view in mind it is possible to conclude in retrospect, that

yeast-gum, the first polysaccharide ever to be isolated from noncapsulated cells as a soluble specific substance [Mueller & Tomcsik (97)], is located at the surface of the cell wall, since Tomcsik (98), recognizing the type specificity of this substance, demonstrated that the agglutination of different *Saccharomyces* types could be elicited with the yeast-gum precipitating antibody.

The indirect immunochemical evidence, obtained by agglutination reactions, regarding the topography of the Vi, O, and R antigens, has been reviewed among others by Morgan (99) and Spooner (100). Thus only a few data will be given here indicating certain principles. The dark-field studies of Pijper (76, 77, 78) permitted certain conclusions concerning topography even on a morphological basis. The bacteria agglutinated by O antisera appeared in the form of a crystalline structure, revealing a characteristic polar attraction. It is therefore likely that the O antigen is concentrated and exposed at the polar surface. A polar concentration of polysaccharide material was, indeed, demonstrated by Lankford, Hoyo & Lutteringer (101) in seven genera of *Enterobacteriaceae* by using the periodate-Schiff polysaccharide stain, whereas rough, O inagglutinable variants selected from smooth parent cultures did not possess stainable polysaccharide. The dark ground studies on Vi agglutination revealed on the other hand a parallel structure indicating the surface location of the Vi antigen on the lateral cell wall (77). Untreated suspensions of young cells are only agglutinable by Vi antibody, whereas partly degraded bacteria may be agglutinated by both Vi and O antibodies; this indicates the surface position of the Vi antigen even relative to O antigen. Morgan (99) concludes on the basis of agglutination reactions carried out with antibodies prepared either against the whole O-complex or against its protein component, that in *Shigella* the polysaccharide and not the protein and phospholipin components are exposed on the surface. The O-complex, which probably forms the superficial layer of the cell wall, disappears during the course of R variation and the R forms of *Salmonella* and *Shigella* give considerable cross reaction (100). Similar conclusions can be drawn from the important work of Miles & Pirie on *Brucella* (102). According to Stacey (103), the R forms are able to produce only a part of the cell wall, which contains more protein. This conception is in agreement with the observation of White (104) that the R forms react more strongly with Millon's solution but give a weaker Molisch test than the S forms. On the contrary, Yoshida *et al.* (105) assume that in *B. subtilis* a protein component is situated at the outer and a polysaccharide in the inner layer of the cell wall. They base their assumption on electron micrographs of isolated cell walls which show a visible change of the exterior surface after treatment with pepsin or trypsin. The immunochemical evidence at present available speaks against this observation.

A new direct approach was attempted by Tomcsik & Guex-Holzer (106) by applying the agglutination reaction on the separated cell walls and protoplasts of *Bacillus M.* The cell-wall preparations were obtained by trypsin

digestion of the bacteria and the protoplasts isolated following controlled lysozyme digestion of the cell wall. Antibodies for these two components were prepared in rabbits. A distinct difference was found between the cell wall and the protoplast antibodies in direct agglutination as well as in absorption tests. The cell-wall antibodies could be absorbed by isolated mucoproteins or mucopolysaccharides but not with the protoplasts. The protoplast antibodies behaved in the reverse way in absorption tests. The cell-wall agglutinates of *Bacillus M.* showed, contrary to *Salmonella*, a variable, polar and lateral attachment (Plate III, Fig. 23). We believe that the direct agglutination of the cell walls offers a technically simple approach to the study of the topography of bacterial antigens. Preparation of cell walls in the Mickle disintegrator can easily be applied to different species of bacteria although, according to our observation, the prolonged shaking may decrease the stability of the bacterial suspension. In this case a complement-fixation reaction may be applied.

Cell wall and protoplast antibodies have been prepared recently also by Yoshida *et al.* (107) against *Hemophilus pertussis* and *Vibrio cholerae*. The two cell components were separated from each other by the agitation method, and it was found that the cell-wall antibodies of *H. pertussis* were effective in the protection-test, and in the presence of complement those of *V. cholerae* dissolved the homologous cell-wall preparations.

SEROLOGICAL REACTIONS OF THE PROTOPLASTS

Knaysi (108) and Bisset (75) listed a number of cytological arguments to prove that the cytoplasm of the bacterial cell is surrounded by a definite morphological entity, the cytoplasmic membrane. Bisset (74) states that "the behaviour of the membrane at cell division indicates that it is a positive structure, bounded upon each side by a surface, and it is not merely an interface between the cytoplasm and the cell wall." Mitchell (109) assigns the location of the osmotic barrier to the "so called cytoplasmic surface" and estimated its thickness at about 10 μ . This may also be considerably larger, if part of it is occupied by a supporting structure. As he states, the cell wall and the cytoplasmic membrane of Gram-positive bacteria cannot be separated by osmotic forces, hence there is some doubt about the situation of the osmotic barrier in these bacteria. Stähelin (110, 111) on the contrary, obtained a clear cut separation of cell wall and cytoplasmic membrane in a vaccine-strain of *B. anthracis*. This strain revealed probably a hereditary weakness of the cell wall leading to a spontaneous extrusion of the protoplasts in wet preparations; this extrusion was increased by changing the osmotic concentration of the suspending fluid. The last effect led also to the fusion as well as subsequent separation of the free protoplasts indicating the presence of a membrane.

The separation of the cell wall from the cytoplasmic membrane was first demonstrated by the use of enzymes by Tomcsik & Guex-Holzer (41) on *Bacillus M.* As described previously, a controlled lysozyme digestion of these

living bacteria elicited primarily a cell-wall dilatation leading to its separation from the cytoplasmic membrane and to the spherical transformation of the protoplasts (Plate III, Figs. 24 to 26). The survival of the protoplasts, set free after the total dissolution of the cell wall, necessitates in most strains of *B. megaterium* the application of 0.1 to 0.2 *M* sucrose solution as the suspending fluid as demonstrated by Weibull (45). The protoplasts of *Bacillus M.* survive partially in 0.85 per cent NaCl solution and fairly well in Ringer solution (112). When the bacteria were suspended in physiological saline it could be observed in phase contrast that some of them suddenly faded during spherical transformation (41). Their homogeneous black appearance changed; they became pale, leaving behind a well visualized "ghost" corresponding morphologically to the assumed cytoplasmic membrane. Weibull (113) separated these elements from the cytoplasm by centrifuging the suspension obtained through dissolution of the protoplasts in phosphate buffer, at between 590 and 14,800 G. On the basis of absorption spectra of the cytoplasmic membrane, he located the entire cytochrome system in them. Furthermore he could demonstrate the existence of a permeability barrier at the surface of the free protoplasts (114).

Tomcsik & Guex-Holzer (106) used the free protoplasts of *Bacillus M.* (Plate III, Fig. 27) after washing several times in sucrose solution, to produce antibodies by 10 intravenous injections in rabbits. This antibody agglutinated the protoplasts in 1:80 to 1:320 dilution (Plate III, Fig. 28) but it did not agglutinate the cell-wall suspension. The cell-wall antibody behaved in the reverse way. Since an agglutinated action of the protoplast antibody on autolyzed protoplasts was also observed (Plate III, Fig. 29), Tomcsik & Baumann-Grace (43) studied the effect of this antibody on the isolated and washed cytoplasmic membranes. By using the agglutination as well as the complement-fixation reactions, they observed that the cytoplasmic membrane behaves serologically in a similar way to the whole protoplast. The nature of the serologically active substance and an eventual serological difference between the cytoplasmic membrane and the cytoplasm have not been worked out as yet. Preliminary results speak rather for the occurrence of an identical component both in the cytoplasmic membrane and in the cytoplasm.

Difficulties arose also in the interpretation of the action of protoplast antibody on the living intact and on the heated cells of *Bacillus M.* The former are agglutinated by 1:640 to 1:1280 dilutions of the anti-protoplast, as well as the anti-cell-wall serum and the latter only by the cell-wall immune serum (106). This observation indicates that on the surface of living non-capsulated *Bacillus M.*, obtained from aerated bouillon culture, thermolabile protoplast material is exposed in some unknown way. As yet we have not been able to ascertain whether this material corresponds to the flagellar substance. Since the isolated protoplasts may retain their flagella (45), they may be able to induce the production of flagellar antibodies as well. Very recently, we observed, indeed, certain differences in the reaction of protoplast

antibodies with intact bacteria as compared with the protoplasts proper.

Is it at all conceivable that protoplasmic material or the cytoplasmic membrane could be exposed freely in some places on the surface of intact bacteria? We know too little about the finer construction of the bacterial cell wall to consider the possibility of such a conclusion seriously. Nevertheless there are certain observations indicating a mosaic-like structure of the bacterial surface. Johnson, Zworykin & Warren (115) observed transparent areas of mosaic-like individual units, visible in the electron microscope, on the cell wall of luminous bacteria after their disruption by osmotic means. The diameters of these circular or elliptical units varied between 5.6 and 8.4 μ . Houwink (116) gave an extremely clear demonstration of the double structure of the cell wall in a *Spirillum* species. The outer part consisted of two or three intercrossing fibril-like layers or else of globules arranged in a hexagonal pattern. The inner layer appeared to be smooth. Since he obtained the cell walls by crushing the cells in a Mickle microshaker, the question might be posed also here whether his preparation was free from cytoplasmic membrane. Yoshida *et al.* (72) also assumed a mosaic-like structure of the cell-wall surface in *S. typhi*. Tomcsik & Baumann-Grace (43) recently studied the agglutination of intact cells of several freshly isolated soil strains of *B. megaterium* by homologous and heterologous protoplast immune sera. Contrary to the case with *Bacillus M.*, they did not find a positive agglutination indicating an exposed protoplasmic material, though the bacteria were obtained, similar to *Bacillus M.*, from aerated bouillon cultures. The direct agglutination and complement-fixation tests carried out with the isolated protoplasts revealed, however, a serological similarity between these elements. We think that such studies might be of use in throwing light on the phylogenetic relationships of certain bacteria.

Yoshida *et al.* (107) also prepared immune sera in rabbits by using as antigens the dissolved bacterial substances, obtained by shaking living bacteria and removing the cell wall by centrifuging the suspension. They regard these as anti-cytoplasmic immune sera. The serum prepared with *H. pertussis* cytoplasm afforded no protection against infection, and that prepared with *V. cholerae* cytoplasm did not exhibit a bactericidal effect, in contrast to the actions of the cell-wall antisera.

In this review, rather isolated observations regarding the possibilities of using antibodies as cytological indicators have been discussed. The author believes, however, that the relatively simple methods described here may be extended to a larger variety of microorganisms and may bring useful results in elucidating further the structure of the bacterial cell. He expresses the hope that the more complicated technique of using labelled antibodies, reviewed recently by Coons (117), may amplify this research.

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POX VIRUSES¹

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In his review of classification and nomenclature of viruses, Andrewes (1) set down the properties associated with pox viruses. Electron microscopy indicates that typical members of the group are brick-shaped. All are rather large and tend to be resistant to physical agents and to chemicals such as ethyl ether. Some members exhibit immunological cross-reactions. Most are transmitted as a result of entry through the skin or perhaps mucous membranes. Many cause cell proliferation as a preliminary to cell necrosis and most form cytoplasmic inclusion bodies. All induce skin lesions often of the "pock" type beginning as a papule or vesicle. The classification along Linnean lines proposed by Holmes (2) and modified by van Rooyen (3) has not been generally accepted. For the time being, the group name Poxvirus has received official approval (4) and the following names have been accepted for members of the group; *Poxvirus variolae* (smallpox), *Poxvirus officinale* (vaccinia), *Poxvirus bovis* (cowpox), *Poxvirus muris* (ectromelia), *Poxvirus avium* (fowlpox), *Poxvirus mollusci* (molluscum contagiosum), and *Poxvirus myxomatis* (rabbit myxoma). The criteria for inclusion in the group are: (a) large particles of rounded quadrangular form with longest diameter in the range 200 to 350 m μ ; (b) predilection for infection of skin epithelium with the production of cytoplasmic inclusion bodies in a proportion of the cells (5). Other animal viruses included in the pox group by Goodpasture (6) and Buddingh (7) are the viruses of sheep pox, goat pox, horse pox, and swine pox. Although examination of these by the electron microscope has not yet been recorded, in other respects they possess most of the characters of the pox group.

The virus subcommittee at the Fifth International Congress of Microbiology held at Rio de Janeiro in 1950 agreed upon eight principles on which the classification of at least the animal viruses should be based (1). In the present review the material has been considered on the basis of these principles and, in the final section, relationships within the group are discussed. Because of the limited space allotted to this review little reference has been made to clinical features, epidemiology and specific immunization. The papers cited have been restricted in number, perhaps somewhat arbitrarily; and no attempt has been made to deal fully with those aspects of the subject which have been covered in recent reviews on related topics.

MORPHOLOGY AND METHOD OF REPRODUCTION

The viruses of this group are large enough to be visible with the light microscope. The relation of elementary bodies to inclusion bodies was dem-

¹ The survey of literature pertaining to this review was concluded in December, 1955.

onstrated many years ago by Woodruff & Goodpasture (8), by Barnard & Elford (9), and by van Rooyen (10). These results and observations made on the development of pox viruses in the living cell [Himmelweit (11); Bland & Robinow (12)] have largely been confirmed by more recent work with the electron microscope.

The phase contrast microscope has provided a new method of demonstrating pox viruses. In "dab" preparations from infected tissue the appearances could be compared with those of the same fields in stained preparations [Herzberg & Bommer (13)]. Stoeckenius (14) enhanced the contrast of his images by altering the suspending medium.

Since the first papers on electron microscopy of vaccinia, ectromelia, and myxoma virus [Borries, Ruska & Ruska (15); Ruska, Borries & Ruska (16)], techniques have been improved and other viruses of the pox group examined. Excellent reviews of the techniques used, the results obtained, and the difficulties of interpretation have recently been published [Williams (17); Bang (18)]. The pox viruses examined in dried films are variola (19, 20), canary pox (21, 22), fowlpox (23, 24), molluscum (21, 23, 25), ectromelia (21, 23), myxoma (21, 26), and fibroma (27). In such preparations all the viruses examined reveal brick-shaped bodies mostly within the size range $250 \text{ m}\mu \times 200 \text{ m}\mu$ to $250 \text{ m}\mu$. Greater variation is shown in estimates of the height of the particles in shadowed preparations. The sizes are of the same order as those determined by filtration and centrifugation studies. The brick-shape may be partly attributable to flattening and distortion caused by the methods used.

There is considerable evidence of an internal structure in the pox virus particle. Green, Anderson & Smadel (28) noted a central area more opaque to the electron beam than the periphery of the particles. Short treatment with 0.02 N NaOH increased the prominence of the central area. Stronger alkali left empty ghosts with the appearance of a peripheral membrane. Dawson & McFarlane (29) showed that pepsin treatment of vaccinia virus removed much of the phosphorus and deoxyribonucleic acid. Deoxyribonuclease liberated nucleic acid in soluble form with some loss of density of the central body. Similar observations have been made on fowlpox (30). Peters & Nasemann (31) described degrees of digestion of vaccinia virus according to the method of fixation used prior to the action of pepsin. Peters & Stoeckenius (32, 33) stressed the value of Chabaud fixation prior to digestion. The inner body left after treatment with pepsin was not removed by deoxyribonuclease, but when this enzyme was followed by further treatment with pepsin the inner body disappeared leaving an empty membrane. The observation that 0.01 N HCl alone may help digestion of the inner body of molluscum was held to indicate the presence of histone (34). Peters & Wigand (35, 36) have shown that *Escherichia coli* and *Neisseria sicca* present similar appearances following peptic digestion, and it is suggested that pox viruses have essentially the same organised structure as bacterial cells (37).

The recently developed techniques of preparing ultrathin sections from tissue embedded in methacrylate have added to our knowledge of the intra-

cellular forms of pox viruses, though the problems of artifact production are considerable (17, 18). Many forms, all oval or round, attributable to virus particles have been described varying in size, density and inner structure. These have been interpreted as a regular sequence of developmental forms arising, in the case of vaccinia, from a matrix near the nucleus of the cells [Gaylord & Melnick (38)]. Morgan and his colleagues (39) have produced excellent electronmicrographs of cells infected with vaccinia and fowlpox. They suggest that the virus particles develop from aggregates of granular material within the cytoplasm, which correspond to the inclusion bodies seen under the light microscope. Some particles appeared to differentiate as individual units scattered through the cell. Many particles had incomplete membranes and before release from the cell often showed a double membrane. Neither Gaylord & Melnick nor Morgan *et al.* report sequential studies designed to follow the course of infection, and thus, lacking correlation with biological observations, their schemes of developmental cycles must remain conjectural [Schlesinger (40)]. Pox viruses which have been examined in thin sections of infected tissue include fowlpox (30, 39, 41, 42), vaccinia (25, 38, 43, 44), molluscum (38, 43, 45, 46), ectromelia (38), myxoma (47), and fibroma (27).

An apparent eclipse "phase" has been observed with several viruses of the pox group. Studies with vaccinia virus on the chick chorio-allantois have been reported by Briody & Stannard (48), Anderson (49), Fenner (50), and Lepine *et al.* (51). Similar eclipse periods have been reported with vaccinia in rabbit skin by Crawford & Sanders (52) and with myxoma virus in the chorio-allantois by Fenner (50). The explanation of this eclipse phase is not clear but it seems premature to infer that the "lost" virus has broken down within the cell into nonliving units preparatory to the synthesis of new viral materials.

CHEMICAL COMPOSITION

The validity of chemical analyses of viruses depends on the purity of the elementary body suspensions used. With purified suspensions in which the ratio of number of particles to number of infectious units was 4.9:1, it has been shown that the percentage composition in dry weight of vaccinia virus is: carbon 33.7; nitrogen, 15.3; phosphorus, 0.57; copper, 0.05; cholesterol, 1.4; phospholipide, 2.2; neutral fat, 2.2; reducing sugars, 2.8; thymonucleic acid, 5.6 [see Smadel (53)]. Of these, cholesterol can be removed by ether extraction without altering the infectivity of the preparation. Vaccinia elementary bodies also contain biotin and flavin. Phosphatase, catalase, and lipase activity were found, but these might well have been absorbed from the host cells (53). Information in similar detail is not available for other pox viruses.

SUSCEPTIBILITY TO CHEMICAL AND PHYSICAL AGENTS

The pox viruses generally are resistant to drying but, when freed from protective protein, are readily inactivated on exposure to ultraviolet light. They are resistant to phenol and some of the common disinfectants but sus-

ceptible to oxidising agents such as potassium permanganate [see van Rooyen & Rhodes (54)]. Since the relative resistance of vaccinia and ectromelia to the action of certain surface-active agents was demonstrated (55, 56), Andrewes & Horstmann (57) have used ether susceptibility in an attempt to assist virus classification. They found that vaccinia and ectromelia were resistant, while myxoma virus was relatively susceptible. Bennett, Horgan & Haseeb (58) reported that goat pox and sheep pox viruses were resistant to ether. Fenner (50) reported some data on the inactivation at 55°C. of various pox viruses in normal serum. Myxoma and fibroma viruses were more rapidly inactivated than ectromelia, fowlpox, cowpox, and vaccinia. Many other observations on the resistance of pox viruses are available, chiefly in connection with the preparation and keeping qualities of vaccines. Crude preparations have often been used, and the results are not germane to the present review.

Chemotherapy.—The subject of chemotherapy of virus infections has been reviewed by Hurst (59) and more comprehensively by Matthews & Smith (60). Most of the experimental work on chemotherapy in the pox virus group has used vaccinia infection of tissue cultures, chick embryos, and mice as model systems. Some inhibitory effect has been shown by certain analogues of purines, pyrimidines, amino acids, and vitamins, by some acridine compounds [Thompson and his co-workers (see 60)] and by thiosemicarbazones [Bauer (61); Hamre, Brownlee & Donovan (62); Thompson and co-workers (see 60)]. Groupe & Rake (63) found an impurity in commercial penicillin which inhibited canary pox, fowl pox, and vaccinia. The results obtained so far with pox virus infections can only be regarded as a preliminary prospecting of the field. They indicate the possibility of learning more of the intracellular life of these viruses and the eventual development of practical chemotherapy.

IMMUNOLOGICAL PROPERTIES

Vaccinia was the first of the animal viruses to be obtained in relatively large quantities as a purified suspension of elementary bodies, and its antigenic structure and serological reactions have been studied for many years. The results up to 1942 are reviewed by Smadel & Hoagland (64) and Smadel & Shedlovsky (65), and there is a more recent account by Smadel (53).

Vaccinia virus has several antigenic components. There is a soluble antigen (LS), which, though apparently a single protein of molecular weight 240,000, has yet two reactive fractions, one heat labile, the other not. Half the substance of the virus particle is made up by a nucleoprotein antigen (NP). Inactive virus or the separated LS and NP antigens stimulate the production of antibodies in rabbits. These antibodies react *in vitro* with the antigens and agglutinate suspensions of elementary bodies, but have little or no protective effect. A rather better protective antibody response may be elicited in rabbits and monkeys by ultraviolet-irradiated vaccinia virus [Collier, McClean & Vallet (66)], but this is still much poorer than the response to infection. Neutralising antibody is readily absorbed from potent

antivaccinal serum by living virus but not by the separate antigenic extracts [Salaman (67)]. It would appear that any method so far used to inactivate the virus degrades the antigen necessary to stimulate the formation of neutralizing antibody and effective immunity to infection.

The antigens of other viruses of the pox group have not been studied in such detail. The LS antigen extracted from crusts of human variola is serologically indistinguishable from the LS antigen of vaccinia [Craigie & Wishart (68)]. This is the basis of the serological tests used in the diagnosis of suspected smallpox cases. Convalescent sera from alastrim and smallpox cases reacted equally well with soluble egg antigens of the viruses of variola major, alastrim, vaccinia, and cowpox [McCarthy & Downie (69)]. The slight difference between the soluble antigens of vaccinia and cowpox (70) has recently been confirmed by Gispén (71), who used the method of double diffusion in agar gels. His results suggest that there is a quantitative difference in a shared antigen. A marked serological similarity was found between variola, vaccinia, cowpox, and ectromelia viruses by the direct and indirect complement fixation techniques (72). The elementary bodies and soluble antigens of myxoma virus, obtained from infected rabbit skin or chick chorio-allantois, react specifically with antimyxoma or antifibroma sera but not with antivaccinal sera [Rivers & Ward (73); Teixeira & Smadel (74)]. From the lesions of molluscum contagiosum a soluble antigen can be obtained which fixes complement specifically with occasional sera from patients [Mitchell (75)]. This antigen does not react with antisera to vaccinia, cowpox or fowlpox viruses.

Vaccinal haemagglutinin (HA) was first described by Nagler (76). Only some fowls have agglutinable cells and the proportion varies considerably with the breed [Suzuki *et al.* (77)]. Vaccinal HA is distinct from the virus particles [Burnet & Stone (78)]; it has a phospholipid and a viral component and can be specifically inhibited by immune sera (79). Chu (80) found the HA to be heat stable, associated with particles of 65 μ diameter and distinct from LS antigen. It failed to fix complement with antibody to LS and anti-haemagglutinin did not neutralize virus. Gillen, Burr & Nagler (81) found two fractions of HA. Their heat stable fraction sedimented with the virus particles at 17,000 r.p.m. leaving a heat labile fraction in the supernatant. Briody (82) confirmed that HA could be separated in this way, but because of the results of treatment with trypsin and ethyl alcohol he suggested that HA was polydisperse. Tagaya's observations (83) support the view that the heat stable fraction of HA is not bound to the virus. Vieuchange & Giuntini (84) showed, by electron microscopy of agglutinated fowl red cells, that the virus particle plays no part in the agglutination. The estimation of anti-haemagglutinin in sera has been used to follow the antibody response to vaccination (85 to 88), and as a diagnostic test in smallpox (89).

North (90) found that an HA serologically similar to vaccinal HA could be obtained in low titre from chick chorio-allantoic membranes infected with variola virus. Burnet & Boake (91) reported that extracts of chick embryos

and organs of mice, infected with ectromelia, contained an HA similar to vaccinia HA except that it agglutinated mouse red cells as well as fowl cells. McCarthy (92), however, finds that mouse red cells are agglutinated both by vaccinia HA and by ectromelia HA.

Cross immunity and neutralization tests.—Cross immunization experiments may fail to bring out minor differences between related viruses. Moreover, to obtain reliable results a common susceptible host is desirable. A more delicate, though indirect, technique for this purpose is provided by the examination of antisera to the viruses in question by *in vitro* or *in vivo* tests. The neutralization test may be carried out in a common host when such is available, or, with suitable controls, in the natural hosts of the viruses under study. Added precision may be obtained by the use of absorbed sera.

Cross immunity experiments in monkeys have shown the reciprocal immunity induced by strains of variola, alastrim, and vaccinia [Horgan & Haseeb (93)]. Burnet & Boake (91) showed that injections of living ectromelia virus would actively immunize rabbits against vaccinia and vaccinia virus, in its turn, immunized mice against ectromelia. This latter observation has been repeatedly confirmed. Many of the pox viruses produce lesions on the chick chorio-allantois and can conveniently be compared on this tissue by using the neutralization technique. The sera of fowls hyperimmunized with the viruses of smallpox, alastrim, vaccinia, cowpox, and ectromelia showed considerable cross neutralization. Examination of the sera after cross absorption with suspensions of the living viruses (prepared from infected chorio-allantoic membranes) showed that smallpox and alastrim are indistinguishable. There were, however, minor differences between these and the other three, which differed from each other [Downie & McCarthy (94)]. It may be noted here that these serologically related viruses are the only ones in the pox group in which HA production has been reported. Neutralization tests on the chorio-allantois provide a convenient method for estimating antibody in post-vaccination and smallpox convalescent sera. For this purpose variola virus is particularly suitable as it produces smaller lesions and later secondary lesions than vaccinia (95, 96, 97).

The avian pox viruses appear to be closely related immunologically to each other but unrelated to vaccinia. Strains from different sources seem to vary in their pathogenicity for different species of bird. Immunization tests by Beaudette & Hudson (98) showed that chickens immunized with turkey pox or fowlpox virus were immune to challenge 33 days later with these two viruses and with canary pox and pigeon pox. Birds immunized with canary pox or pigeon pox, however, were immune to these two viruses but showed less protection against the other two. The canary and pigeon pox viruses produced mild immunizing infections. This may have accounted in part for the lower degree of immunity produced by them against the more virulent fowl and turkey strains. The chickens immunized and challenged with canary pox were thereafter immune to fowlpox. De Blicke (99) has stated that the strains responsible for infection in fowls, pigeons and canaries are

distinct but that the position of turkey pox is uncertain. All are, however, immunologically related.

There seems to have been little recent work on the immunological status of other animal pox viruses and much of the older literature is confusing. Swine pox virus is immunologically unrelated to vaccinia [Shope (100)]. Shope suggests that confusion may have arisen in the past because swine are susceptible to vaccinia and infection with this virus may have been confused with true swine pox. The virus of sheep pox appears to be immunologically unrelated to the other animal pox viruses, except possibly to goat pox [Angeloff (101)]; but Bennett, Horgan & Haseeb (58), working in the Sudan, were unable to establish the virus of sheep pox in goats, nor did goat pox virus immunize sheep against sheep pox.

NATURAL TRANSMISSION

From the infected host, pox viruses are discharged to the environment from the lesions on the skin and mucous membranes. Infection of new hosts occurs in most instances directly or indirectly through the skin or exposed mucous surfaces. The survival of the virus in dried exudate or in dust may, in some instances, facilitate infection by inhalation. Many of the pox viruses appear to be able to survive in an inactive state within the herd, for there are many recorded instances of epidemics being started off by non-specific stimuli. Kikuth & Gönner (102) record, for example, the initiation of an ectromelia epidemic during mouse passage of influenza virus. Some among the pox viruses have been shown to be transmitted by means of insect vectors acting as mechanical carriers. Thus myxomatosis may be transferred in Australia by mosquitoes [Fenner, Day & Woodroffe (103)], and in England by rabbit fleas [Allan & Shanks (104)]. The avian poxes have been shown to be carried mechanically by mosquitoes [see Cunningham (105)]. Swine pox epidemics are intimately connected with lice and can be aborted by delousing a herd [Shope (100)]. Cowpox as a disease of domestic animals has favourable opportunities of spreading by way of the hands of the milkers, who not infrequently develop lesions on exposed skin. Some outbreaks of clinical cowpox have been shown by virological studies to be derived from human vaccinia [Dekking (106)].

HOST, TISSUE AND CELL TROPISMS AND PATHOLOGY

Most of the mammalian pox viruses are restricted in their range of host species under natural conditions. Under experimental conditions the range may be wider, particularly when very young animals are used. The chick embryo is particularly suitable for experimental work as it is susceptible to all the avian and mammalian pox viruses except those of molluscum, sheep, goat, and swine pox.

Smallpox and alastrim are normally exclusively human infections, but monkeys have been found infected with variola [see Blaxall (107)], and they are also susceptible under experimental conditions. Vaccinia and cowpox,

unlike most pox viruses, can infect a wide range of mammals and accidental infection of man occurs with both. Molluscum infection is strictly limited to man and has not yet been propagated experimentally in other hosts. Ectromelia is found only in the mouse, although inapparent infection may be produced in the rat and possibly the rabbit and guinea pig [see Fenner (108)].

Myxoma and fibroma are normally confined to the rabbit, though Kilham, Herman & Fisher (109) have recorded a natural infection of grey squirrels with a virus closely resembling that causing fibroma in the rabbit. Hares appear to be insusceptible to experimental infection with myxoma virus [Jacotot, Vallée & Virat (110)], but it has been propagated through 30 passages by intracerebral injection in day-old mice [Andrewes & Harisijades (111)]. Older mice were insusceptible.

There have been conflicting reports about sheep pox, but more recent work suggests that it cannot produce progressive infection in other animal species [Bennett *et al.* (58)]. Rao's claim (112) to have cultivated sheep pox on the chorio-allantois has not been confirmed [Ortenzi & Tiecco (113)]. Bennett *et al.* (58) transmitted goat pox to sheep, calves, rabbits, and monkeys. Swine pox produced no obvious infection in calves or rabbits although it persisted locally for some days after intratesticular injection into rabbits [Shope (100)]. The avian poxes are found in several species of birds under natural conditions. The virus isolated from any one species is capable of infecting other species of birds under experimental conditions, though the degree of pathogenicity varies [see Cunningham (105)]. None are pathogenic to mammals and all can be propagated in the chick embryo. Their interrelation will be discussed later.

With the exception of molluscum, and perhaps cowpox, all the pox viruses produce generalized infection in their natural hosts. The involvement of tissues other than skin and mucous membrane can be readily demonstrated in both natural and experimental infections, though the epithelial tissues seem to provide particularly favourable conditions for multiplication. Fibroma virus, which seems to have less predilection for epithelium, produces inclusions in skin epithelium of rabbits [Ahlström (114)].

Fenner (108) and Fenner & Woodroffe (115) have outlined the course of infection taken by ectromelia and infectious myxomatosis; and it seems likely that this is typical of other generalized pox diseases such as smallpox. The recent study of Bras (116) on the morbid anatomy and histology of smallpox, based on a careful study of a large number of fatal cases, provides valuable data on the pathology of the human pox infection. Most of the patients had been treated with antibiotics so that the pathological findings were not complicated by secondary bacterial infection. In general, all pox viruses produce the same type of tissue and cellular change. In molluscum, myxomatosis, and the avian pox infections, hyperplasia is more pronounced than in some of the other pox diseases. The degree of inflammatory reaction varies even with different strains of the same virus or the same strain in dif-

ferent hosts. The intense polymorph infiltration associated with pustule formation in smallpox seems to be part of the response to local virus activity; it occurs in the absence of secondary bacterial infection.

The gross changes in infected cells follow a common pattern, but there are differences in the specific intracytoplasmic inclusions characteristic of the group as a whole. Intranuclear inclusions have been described in the lesions of smallpox and alastrim by Torres & Teixeira (117) and Torres (118), who thought the cytoplasmic inclusions in the two conditions had different appearances. Their observations have not been confirmed in studies of the lesions in human skin or in the chorio-allantois (96, 119). In variola and vaccinia the cytoplasmic inclusions, except in corneal epithelium, appear as less strongly acidophilic, irregular, granular masses, and seem in mature forms to be composed of masses of elementary bodies with relatively little matrix. On the other hand, because of the greater amount of matrix material, the intracytoplasmic inclusions of molluscum, fowlpox, cowpox, and ectromelia are frequently large, strongly acidophilic, homogeneous in appearance, and sharp edged. Their appearance varies with the stage of infection, the experimental host, the tissue examined, and the methods of fixing and staining. They have been separated by digestion and micromanipulation from the rest of the cell and with the electronmicroscope sometimes appear to be sharply separated from cell cytoplasm. It is doubtful whether, in molluscum, the virus particles develop from the trabeculae of the inclusion matrix, as has been suggested by Melnick *et al.* (25). The histochemical studies of Rake & Blank (120) on sections of molluscum bodies indicated the presence of ribonucleic acid in early inclusions which was compressed by increasing amounts of deoxyribonucleic acid in more mature bodies, a finding which confirms results obtained by microspectrographic methods [Hydén (121)]. The inclusions of fowlpox were found by Goodpasture to stain with fat stains. Their reaction to treatment with osmic acid, ether, and sodium lauryl sulphate [Bang *et al.* (30)], and to Russell viper venom [Lepine *et al.* (122)] suggests that the matrix may be lipoprotein in nature. Histochemical study of fibroma inclusions suggested to Fisher (123) that here the matrix material was of glycoprotein nature. Multiplication of virus may occur without the formation of demonstrable inclusions as in chick tissue cultures infected with fowlpox (30). Vaccinia virus particles may be found in the cytoplasm outside inclusions, or before inclusions are demonstrable (39). The exact relationship of the inclusion material to the disordered metabolism of the cell and to the multiplication of virus within the cell are matters which await elucidation.

RELATIONSHIPS WITHIN THE POX GROUP

As has been previously indicated, the viruses of smallpox, alastrim, vaccinia, cowpox, and ectromelia are closely related immunologically.

Variola and alastrim.—These viruses are stable in that the diseases, although clinically alike, differ constantly in mortality in unvaccinated individuals. The strains gave almost complete cross protection in experiments in

monkeys; the slight differences obtained were probably attributable to differences in the virulence or size of dose of the immunizing strain [Horgan & Haseeb (93)]. The same factors probably account for minor differences among strains of alastrim tested in the same way [Horgan, Haseeb & Satti (124)]. Immune sera prepared in fowls against five strains of smallpox virus and one strain of alastrim failed to differentiate these viruses by neutralization tests in the chorio-allantois (94, 97). The difference in virulence for man of alastrim and smallpox viruses is not reflected in their antigenic structure, but is paralleled by difference in the mortality rate in chick embryos [Helbert (125)].

Cowpox.—The strains isolated from the spontaneous disease in cattle show definite differences from laboratory strains of vaccinia [Downie (70, 126, 127); Dekking (128); Verlinde (129); Herrlich & Mayr (130)]. Nine strains isolated in England in recent years from cows, or persons infected from them, all showed the characters of cowpox virus. Infection in cows with vaccinia virus is sometimes contracted from a recently vaccinated milker. Of 36 strains recently isolated from outbreaks of cows or associated human infections in Holland, 28 were typical cowpox strains and 8 were vaccinia [Dekking (106)]. Generalised infection in an eczematous child and demyelinating encephalomyelitis, both of which may follow vaccination, have also resulted from cowpox infection [Verlinde (129)]. Cowpox virus propagated on the chorio-allantois readily gives rise to a stable variant. This produces lesions on the chorio-allantois and in rabbit skin which lack the haemorrhagic character of the parent strains; it is slightly less virulent for the chick embryo and for laboratory animals [Downie & Haddock (131); van Tongeren (132)].

Vaccinia.—Strains of vaccinia from various sources and of diverse origin have been used and studied in many laboratories over many years [see Buddingh (133)]. There is no evidence that such strains vary immunologically and probably all give immunity against variola [Horgan & Haseeb (134)]. They may vary considerably in their tissue tropisms and virulence by different routes of inoculation for animals and chick embryos, probably as a result of varying methods of propagation over many years in different laboratories and lymph institutes [Herzberg (135); Herrlich & Mayr (136)]. Several fixed variants have been recognized in such terms as dermo-vaccine, neuro-vaccine, and testicular vaccine. The designation of any one strain as the "type" strain would seem to present a nice problem to the systematists!

The origin of vaccinia is obscure. Some strains are reputed to have been derived from human smallpox material by passage through laboratory animals. It has often been pointed out that most of such vaccinal derivatives have been produced in laboratories where vaccinia was already under study. That vaccinia is highly infectious for laboratory animals has long been established, and there must in many instances remain some doubt as to the ancestry of such derived strains [see Horgan (137)]. Variola virus propagated in the chorio-allantois over many successive passages retains its typical char-

acters [Buddingh (95); Nelson (138); Downie (127)]. Nor is it easy to effect the transformation from variola to vaccinia by the recommended techniques of animal passage. Recent attempts to convert variola to vaccinia in monkeys and rabbits under strict conditions of isolation have failed to do more than produce a transient survival of variola in the rabbit [Nelson (138); Buddingh (133); Downie (127); MacCallum (139); Craigie (140)].

Vaccinia and cowpox viruses differ from variola virus in that they both have a wide host range under experimental conditions; both may be found in natural infections of either man or cow, and both have a tendency to produce variants. The wide host range, an uncommon feature among the pox viruses, is in favour of vaccinia being derived from cowpox, as is the recent failure to effect the variola to vaccinia transformation. Against this view is the weight of the older evidence regarding the history of current strains of vaccinia and the similarity of the inclusion bodies of variola and vaccinia. Serological evidence does not weigh heavily on either side. Although vaccinia appears to be more closely related serologically to variola than to cowpox, differences are slight; and the innumerable passages of vaccinia by dermal inoculation of laboratory animals might be held responsible for as much serological shift as vaccinia strains show from either cowpox or variola. It seems likely to the present authors that many, if not all, of the current strains of vaccinia were originally derived from cowpox and that the long continued propagation of lymph strains by dermal scarification has led to a decrease of the affinities of cowpox strains for mesodermal tissues. This quality of cowpox strains seems to be responsible for the haemorrhagic character which their lesions exhibit in some laboratory animals.

Ectromelia (mousepox).—This virus meets all the requirements for inclusion within the pox group, and, as noted above, it is serologically related to variola, vaccinia, and cowpox viruses.

Rabbit pox.—Outbreaks of rabbit pox have been reported only in laboratory stock. The disease is apparently highly infectious and attended by considerable mortality. The virus is more virulent for the chick embryo than most strains of vaccinia, with which it appears to be immunologically identical [Rosahn, Hu & Pearce (141)]. It has apparently not been examined by the electron microscope. In view of its similarity to neuro-vaccinia there seems to be insufficient evidence to warrant its designation as a separate, named virus.

Fowlpox, turkey pox, canary pox, and pigeon pox.—The avian pox viruses isolated from different species of birds are closely related and for the time being are best regarded as variants of one virus, designated by the official term of *Poxvirus avium*.

Molluscum contagiosum.—The claim of this virus to be assigned to the pox group rests on the morphology of the virus and inclusion bodies, and the nature of the lesions found in the skin. It has so far proved impossible to infect hosts other than man, and propagation in tissue culture has not been reported. Investigations of other properties have therefore been restricted.

Myxoma and fibroma.—The case for placing these viruses in the pox group has been made by Fenner (50). The virus of myxoma seems serologically unrelated to vaccinia and, as noted above, is more susceptible to inactivation by chemicals.

Goat pox, sheep pox, and swine pox.—There seems need for further study and characterization of these viruses. Although not yet studied by electron microscopy and apparently not related immunologically to other pox viruses or to each other, their clinical and pathological features, the nature of the inclusions, and what is known of the size of the viruses warrant their consideration with this group. Horse pox now seems an uncommon disease, and the virus has not been studied by modern techniques.

Other viruses which should be considered for inclusion in the pox group are the virus of contagious pustular dermatitis of sheep and goats [Horgan & Haseeb (142)] and the virus of milkers' nodes. These two viruses, both of which affect man as well as the usual animal host, are unrelated immunologically to vaccinia [Lloyd, Macdonald & Glover (143); Berger (144)]. The sizes of the virus particles seem to be within the same range as the pox viruses.

"Poxvirus" is a convenient term denoting a number of viruses which have many features in common, though they comprise several immunologically unrelated subgroups. A balanced account of their properties and interrelationships is not possible at the present time as they range from vaccinia, one of the most extensively investigated of all viruses, to others about which little is yet known. It is the authors' hope that this review may have drawn attention to some of the more obvious gaps in our present knowledge of this interesting group.

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CYTOLOGY OF BACTERIA^{1,2}

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INTRODUCTION

In going over the recent literature of bacterial cytology, one is impressed not only by the number of publications but also by the number of languages in which the papers are written. Fortunately, many of the foreign journals require abstracts in English, French, and German. Much of the research is purely cytological, dealing with the bacterial cell and methods of its study, but what is even more pleasing is that the relationships between cytology and other branches of bacteriology are gradually becoming clearer, and one now finds instances in which cytological observations are used to explain physiological or other processes.

The present review covers most of the literature of 1954 and 1955, but papers that appeared before these years are sometimes included, either because they were not covered in previous volumes of this series, or because they are considered essential for the discussion. Because of the limitation of space, and the present status of certain phases of bacterial cytology, it was considered more valuable to be liberal with pertinent discussions of a few selected phases of bacterial cytology than to cover the entire field with a succession of names and numbers. For this reason, a number of topics that are integral and fundamental parts of the field, such as chemical composition of the cell, physico-chemical relations between cell and environment, vegetative reproduction, the endospore, staining procedures, morphologic variation, and colony formation had to be omitted. This involved more than 250 references actually gathered.

The chief reason for the insufficiency of space is that in this series bacterial cytology is not reviewed every year, so that one is confronted with the very difficult task of confining a review to a space hardly enough for a bibliography that would include titles of the papers to be reviewed.

Last year a book on bacterial cytology was published in Italian by Scanga (133). The book is richly illustrated, especially with electron micrographs. It disposes of controversial phases by including both or various views. Modern literature antedating the book is adequately covered. The rickettsiae, viruses, and spirochetes also receive consideration and occupy each a chapter. This book is an excellent sequel to a previous one (55), perhaps the most artistically produced volume on bacteria, which summarizes the work on

¹ The survey of literature pertaining to this review was concluded in December, 1955.

² The following abbreviations have been used in this chapter: DNA (deoxyribonucleic acid); RNA (ribonucleic acid).

microorganisms carried out at the Istituto Superiore di Sanità with the electron microscope.

A review on certain phases of bacterial cytology (106) and another one on the bacterial nucleus (28) appeared in Volume 8 of this series. There was also an article on the "cytoarchitecture" of bacteria by Cutinelli (26).

NUCLEUS

Existence and properties.—To the list of those who undoubtedly observed bacterial nuclei before the modern period of bacterial cytology one should add the name of Mori (102), who gives in this paper a brief account of his earlier studies.

Knöll & Zapf (74) observed with the phase microscope light and dark areas during the phase of growth and division of various gram negative and gram positive bacteria. These areas corresponded, respectively, to the distribution of the nuclei and cytoplasm. The study of normal cultures and of cultures infected with phage, or under the influence of antibiotics, or exposed to supersonic vibrations led the authors to conclude that the alternating light and dark structure is attributable primarily to differences in the RNA-DNA structure of the cell. Krieg (76 to 82) investigated the granular content of the cell in various groups of bacteria, using phase and fluorescence microscopes. He concluded that there are three types of granules: the first, visible with the phase microscope and demonstrable when acridine orange or auramine are used as fluorochromes, are thread-like, spiral, or granular "nuclear equivalents"; the second type are the "paranucleoids," which appear as dark granules with the phase microscope and are equivalent to the nucleoli of higher cells; the third type stain selectively with the respiratory poison berberine, but only *in vivo*. The latter may store polymetaphosphate, purines, and pyrimidine, and in this state become known as metachromatic granules. Maaloe *et al.* (94) found that, contrary to the view of Robinow (128), the nuclear sites correspond to opaque areas when observed with the electron microscope.

Floethmann (39) made a comprehensive study of *Azotobacter chroococcum* using the Feulgen reaction, HCl-Giemsa staining, tetrazolium chloride reduction, and observation of living cells with the phase microscope. He saw in cultures more than one day old, paired, dark bodies corresponding in reaction and behavior to the cell nuclei. In younger cultures he was unable to observe any nuclei. Here again we find the nuclei appearing as light or dark bodies, apparently with the same type of phase contrast, confirming what for several years the reviewer has held to be true, namely, that with the phase as well as the electron microscopes, whether the nucleus appears light or dark would depend on the organism, the stage of development and other environmental factors, and we must now add the state of the nucleus itself, whether the nucleus is primary or compound. This is in harmony with the conclusion of Bringmann (13) that, with the electron microscope nuclei of the enterobacteria appear like clear bubbles, but under certain conditions,

as in the L-forms of *Escherichia coli* and *Proteus*, the nuclear equivalents appear as opaque, dark bodies.

On the other hand, less careful observers considered density as a sufficient reason to doubt the nuclear nature of granules like those in *Mycobacterium*. Brieger *et al.* (11) for instance, reported that Knaysi *et al.* (73) and Ruska *et al.* (131) considered these granules as nuclei, "However, this interpretation conflicts with the view of Robinow & Cosslett (129) that in bacteria the nuclei are electron transparent." More recently, Glauert & Brieger (41) added other objections to a consideration of the dense granules of mycobacteria as nuclei: first, they "do not appear to be essential for the development of the organism" (cf. also 145); second, "they contain metaphosphate and show metachromasia. . . . They are situated between the chromatinic bodies and cannot be identified with mitochondria." The fact that these granules stained red (as does chromatin) by the method of Jacobson & Webb (56) was attributed to their metaphosphate content. No reference is made to the reviewer's work (68) demonstrating that these granules divide, fuse, and have the structure of bacterial nuclei. The claim that these granules are unnecessary for cell development may be answered by referring to the reviewer's recent report (70) of the existence of primary and compound nuclei, as well as by the fact that visibility with the phase as well as the electron microscope is a relative property.

A primary nucleus, or a small compound nucleus, in a dense cytoplasm such as that of a growing cell may not be visible. This should be obvious and should not require experimental proof. However, the recent observations of Shinohara (137) constitute strong evidence that this is the case. This author confirmed the vacuolizing effect of electron bombardment on the dense granules of BCG. He also noted that "granules may appear and vacuolize by the electron bombardment in the homogeneous cytoplasm having no structure." Unfortunately, Shinohara misinterpreted this observation when he stated: "This fact leads us to suspect that the granules of tubercle bacilli observed with the electron microscope might be artificial products of electron beam," although he considered the possibility that the "electron beam may cause a change in the permeability of cytoplasm, letting the structure reveal the image of granules which have previously been invisible." Shinohara & Fukushi (139; see also 138) concluded that the granules "are considered not to be the permanent and integral structures but to be one of the symbols which indicate the viability of these organisms." He does not explain how a structure can be a symbol of viability without being more than an artifact. Density of the cytoplasm of the organism employed by Shinohara is also responsible for the difficulty in discerning vacuoles except when generated by the electron beam. He may not be aware of the fact that vacuoles can also be seen with the light microscope (cf. 61).

In the reviewer's opinion, the blame for the prevailing confusion regarding the bacterial nucleus in general, and the granules of mycobacteria in particular, rests on the shoulders of those who directly and indirectly have

encouraged the setting of erroneous criteria of density and position. To these we recommend study of the valuable contribution of Peters & Wigand (115, see also 162, 163, 164) who by the consecutive use of ribonuclease and pepsin demonstrated dense nuclei in *E. coli* and *Neisseria sicca* with the electron and phase microscopes. Treatment with deoxyribonuclease and pepsin made apparent transparent areas normally occupied by the nuclei. Consequently, the nuclear nature of an intracytoplasmic structure cannot be rejected because that structure appears bright or dark with the phase or the electron microscope. Brightness and darkness do not depend on the density of the structure alone but on the relative density of structure and cytoplasm. Effect of the scattering of electrons seems to be similar to that of refraction. Of this, the reviewer has always been aware (cf. 71).

Methods of staining.—Piéchaud (118) extended his previous work (116, 117) to include three different methods of staining the nucleus without previous hydrolysis. Piéchaud's methods have received little notice outside France, but they have been frequently used by French workers with considerable success. Michail *et al.* (98) stained cultures of *E. coli*, *Salmonella typhosa*, *Salmonella paratyphosa* A and B, and *Proteus vulgaris* by a modified Severi (136) method using May-Grünwald-Giemsa without previous fixation or hydrolysis. Depending on the cell volume, they observed one to four nuclei per cell. Long cells contained three to four spherical or two or three elongated ones. In short cells there were one nucleus or two spherical nuclei sometimes occupying polar positions. Bringmann (12), staining with methyl green to identify DNA and with pyronin to identify RNA, concluded that in old cultures of *Corynebacterium* the polar bodies contain both RNA and DNA. Tronnier (154) did not find these methods sufficiently specific.

Chance (17, 18) added a new method of staining the nucleus. The method consists of several steps, one of which is a brief exposure to HCl gas. Clark & Webb (20, 21), in a study of the mechanism of Chance's crystal violet method of staining the nucleus, found that the substrate of this method is not the DNA but the protein of the nucleus. The method gave the same results as the HCl-Giemsa and the thionine-SO₂ methods. It gave the most uniform results when used without previous fixation or hydrolysis. Perchloric acid gave inconsistent results with all three methods. Onisi & Kato (111) succeeded in removing RNA by treatment with solutions of salts instead of hydrolysis with acids.

Hartman & Payne (50) applied to bacteria the method of Jacobson & Webb (56) for simultaneous demonstration of DNA and RNA in the cell. Delmotte (32) demonstrated nuclei in coliform bacteria either by growing these organisms in broth containing sodium lauryl sulfate or sulfonate (2.5 per cent), or by treating smears with 5 per cent solutions of these chemicals. In the latter case the smears are fixed with alcohol and dipped in the sulfate or sulfonate solution for 2 to 5 min. After careful rinsing with distilled water, the smear is stained for 5 min. with Unna's polychrome blue or for 1 hr. with Giemsa's stain diluted to 2.5 per cent. The microscopic appearance is identical with that given by the HCl-Giemsa method.

Several papers discuss or deal with fixation and fixing agents. As stated above, Michail *et al.* (98) did not fix their material. Guha *et al.* (47) compared chromic acid with OsO_4 .

The direct phase micrograph of bacteria fixed in chromic acid revealed that the regular pattern of darker and lighter zones as observed after OsO_4 fixation was reversed; the nuclear zone in chromic acid fixed bacteria became darker in comparison with the region occupied by the cytoplasm.

Furthermore, the Feulgen-positive bodies appeared thread-like in cells fixed with chromic acid and round in cells fixed with OsO_4 . The effect of fixation was also studied by Murray (107). The question of whether or not fixation is necessary needs further consideration. It is probable that methods that do not require hydrolysis or some other harsh treatment do not require fixation. Indeed, fixation may even be detrimental as reported by Clark & Webb (20). The reviewer has often noted in his own work that fixation was not necessary. However, when one uses a dilute dye solution, fixation often becomes necessary because it kills the cells and renders them permeable to the dye.

Cassel & Hutchinson (14) took it upon themselves to evaluate some of the early methods used by bacterial cytologists to demonstrate the nucleus. They concluded that adequate techniques for this purpose were known at least 20 years before "the current era of investigation" which, according to Cassel & Hutchinson began in 1942. The reviewer (69) had placed the beginning of the new era in 1937. The period between 1937 and 1942 includes the work of Stille (143), Piekarski (119, 120), Delaporte (31), and a summary of the reviewer's work (64) which was reported in greater detail at a symposium held during the meeting of the Society of American Bacteriologists in December, 1941. The detailed report of the reviewer's work appeared in March, 1942. Among the methods tested by Cassel & Hutchinson was the use of acidified methylene blue. The reviewer has repeatedly stated that this method is excellent for certain organisms like *Staphylococcus flavo-cyanus* and *Mycobacterium* regardless of the medium in which they were grown. Most other bacteria have to be grown in a special medium before their nuclei are demonstrable by this method. It seems odd that Cassel & Hutchinson picked *E. coli* and *Bacillus cereus* on which to test this method. We are not aware that these authors either tested the method with the appropriate organism, or used the appropriate medium. Therefore, their criticism of the method is of dubious worth. For a true evaluation of the method the reader may be referred to any of several publications of the reviewer (e.g., 70). Figure 1 represents cells of *S. flavo-cyanus* stained with acid methylene blue.

Bisset (7) called attention to artifacts that may be interpreted as nuclei or nuclear organization. Bisset showed that

In bacteria which exhibit a tendency to the formation of "cytoplasmic granules," similar granules can be produced artificially by the adsorption of DNA of extraneous origin and staining with a modified Feulgen reaction. . . . The phenomenon supports the suggestion that differences in nuclear and cytoplasmic structures may be due to transfer of basophilic materials between different parts of the cell.

At this point we wish to call attention to a statement in Bisset (8). The statement reads:

When classical bacteriological methods of drying or heat-fixing are employed, or when the methods of dehydration and fixation to which bacteria are subjected cause unsuspected shrinkage and distortion, as is often the case . . . , the entire stainable contents of a cell may be mistaken for a nucleus; as for example in a septate *Staphylococcus* (Knaysi, 1941) or in *Caryophanon* by Peshkoff.

Since the statement contains no reference to any experimental work on which it would be based, the reviewer wrote to the author inquiring about the existence of such work. In his reply, Bisset assured the reviewer that the statement, in so far as it refers to the reviewer's work, is one of those unfortunate slips. Indeed, Bisset has recently (5) pointed out that for many years

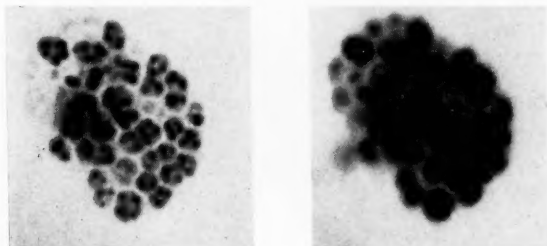


FIG. 1. *Staphylococcus flavo-cyaneus*. A microcolony grown on a collodion film supported by beef infusion, glucose agar. Age 6 hr. and 50 min. at 30°C. Fixed and mounted in methylene blue of pH 3.15 (no other treatment). Left (negative 54-E, 31) shows the nuclei; right (negative 54-E, 29) photographed with different filters to show entire protoplasts.

the reviewer has been aware of the multicellular condition of many so-called single cells.

For the use of fluorescence microscopy, see Krieg (82) and Schuler (135).

Structure and division.—The structure and division of the nucleus are among the most hotly debated questions of bacterial cytology at the present time. In one camp there is the DeLamater school (27, 28) who see in the bacterial nucleus almost a miniature of the nucleus of higher plants and animals, dividing by classical mitosis with centrosome and spindle. In the opposite camp there are most other bacterial cytologists who may not be in agreement among themselves on the structure of the nucleus, but who are unable by any stretch of the imagination to see evidence of mitosis in the division of the bacterial nucleus. A few other cytologists (15, 19) saw evidence of mitosis but did not go to the extreme of describing minute details of all stages.

The most severe critic of the DeLamater school has been Bisset (3, 4, 6) who repeatedly asserted that besides the nuclei, DeLamater's method also

somewhat stains cross walls or cross septa between sister cells. In cells consisting of two or more units, as in *Bacillus* and *Mycobacterium*, the resulting complex picture often superficially resembles a spindle and various stages of mitotic division, the centrioles being represented by the darker spots at the intersections of a cross wall with the peripheral wall, and the chromosomes the nuclei of separate cells. In *E. coli*, considered by Bisset as unicellular,

the centrioles are provided by the material which, in a nonseptate bacterium, corresponds to the septa in *B. megaterium*—i.e. the basophilic areas at the points of division and growing tips of the cell.

Bisset emphasized that cocci also may be multicellular and may present an artificial picture of mitosis in the same manner as *Bacillus* and *Mycobacterium*. Hale (49) reported producing nuclear artifacts resembling DeLamater's mitotic figures by treatment of the cells with organic solvents.

In answer, DeLamater (28) criticized Bisset for inconsistency in explaining the mitosis-like figures he observed in the bacilli. He stated that "the diverse structures described as representing the nucleus are inconsistent and mutually exclusive." In the case of the cocci, DeLamater criticized Bisset for using "parallel preparations rather than simultaneous differential stains on the same cell." He further reported that in an unpublished work Hunter & DeLamater used both the tannic acid method and Hale's method of staining the cell wall and found "that under normal conditions only uni- and bicellular cocci are found. The bicellular state is obviously a division stage and is completely compatible with the nuclear picture described by DeLamater & Woodburn (30). Tetrads arising from large diploid cells by meiosis are found under normal conditions in variable numbers. Whether these groups of four cocci are related to what Bisset calls four-celled cocci is unknown." The paper referred to has now appeared (54).

The reviewer has not had first hand experience with DeLamater's method of staining nuclei and is not in position to give weighty opinion on whether or not Bisset's explanation adequately applies to any or all of DeLamater's observations. He, however, is in position to affirm that Bisset has not been as inconsistent as DeLamater accused him of being. All through his writings Bisset has emphasized that DeLamater's method, like all methods involving fixation and desiccation of the cell, causes considerable shrinkage and distortion of the cell, that it is not absolutely specific for the nucleus but does somewhat stain either septa or cross walls, and that these structures, especially in the early stages of their formation, do appear as granules and have been described as such (48, 62, 66). On the other hand, Bisset has undoubtedly overemphasized multicellularity, for multinuclear cells without evidence of septation are not uncommon. Of course, these are unstable complexes which potentially divide or fragment into uninuclear cells (67, 68).

With this knowledge in mind, it is easy to see that Bisset, in spite of the multiplicity of forms he used to express himself, was consistently referring to the same structures in combination with the nuclei of the sister cells. In the

case of *E. coli*, which he considers unicellular, he indeed reverses the role of the allegedly two stained structures. He now considers the split cross septum to be homologous to DeLamater's chromosomes, and the shrunken nuclei of the sister cells to the centrioles. This interpretation and the one given for multicellular organisms, regardless of their applicability, are not mutually exclusive. The reviewer hopes that his sacrifice of valuable space will clear up any misunderstanding that may exist regarding the position of the principals in this controversy.

Webb & Clark (159) did not observe, but postulated, mitotic and meiotic divisions. Braun & Yalim (10) found that the bacterial nucleus divides directly. Murray & Truant (108) observed a vesicular nucleus with peripheral chromatin granules in *Moraxella*, and Truant & Murray (155) made a similar observation on *Azotobacter*. Yuasa *et al.* (169), studying *Mycobacterium*, found that young cells

elongate after several nuclear divisions and then divide . . . into several individuals, each of which has one nucleus. . . . The nucleus divides according to the type of amitosis. This type of amitosis shows some stage of the evolution to the mitosis.

Fitz-James (36) noted a "continuous increase of DNA coincident with the chromatin separation in germinating spores." This was taken to mean that "a typical mitosis is not occurring" and an "alternative scheme of chromatin duplication" was suggested. Realizing that these results are of little significance unless spore germination is synchronous, Fitz-James (37, 38) later reported that similar results were obtained in cases of nearly synchronous germination.

The reviewer finds little evidence for true mitosis in bacteria, but it seems obvious that a continuous increase in DNA when nuclear division is truly synchronous is unlikely, regardless of the method of division, and that the schemes suggested by Fitz-James to explain the absence of rhythmic increase are based on a nonsynchronous growth and division of the chromatin bodies of the same nucleus. Lark *et al.* (87) did not observe any mitotic patterns in synchronous nuclei of *Salmonella typhimurium*. In *Caryophanon latum*, Tuffery (156) observed a vesicular resting nucleus and rodlike active one, but no mitosis.

Rowen & Norman (130) noted that the length of DNA fibers from various sources, determined by various methods, falls between 0.6 and 1.4 μ . They concluded that in the nucleus of *E. coli*, which is much smaller than 0.6 μ (?), the DNA molecules must be folded; *in vitro*, they unfold. Tulasne & Vendrely (157) reported that in stationary cultures of *Proteus* and *E. coli* the nuclear apparatus consists of a small granule which divides by elongation in the direction of the long axis of the cell. During the logarithmic phase, the nuclear apparatus is in the form of a helix carrying basophilic granules which, while dividing, assume the form of a V, of dumb-bells, etc. In most cases, the nuclear granules result from the aggregation of elementary particles with a mean diameter of 150 m μ , each containing the total complement of genetic factors. Krieg's view on the morphology and structure of the nucleus was reviewed in the previous section.

In a recent report, the reviewer (70) showed that in the bacterial cell there may be one or both of two types of nuclei: the primary nucleus, which is the true carrier of genetic factors, and the compound nucleus which is formed by association of primary nuclei induced by some influence tending to slow down or inhibit growth. The primary nucleus, in its simplest form, consists of a minute mass or granule of chromatin surrounded by a definite membrane. Hyaloplasm is also present and is demonstrable during the division process. Division takes place by elongation of the primary nucleus, separation of the chromatin into two masses, and, finally, cross division and separation of the sister nuclei. Visually, one observes elongation of the chromatin which appears to fill the entire nucleus. After what appears as a transverse division of the chromatin filament, the two resulting masses seem to round up into two terminal beads separated by a clear space probably filled with hyaloplasm. It is at this stage that the nuclear membrane is clearly visible. Sometimes the final division and separation of the daughter nuclei may be delayed until after one or both of the terminal beads of chromatin have divided. This results in the formation of what is called complex, primary nucleus. This, however, is temporary, and complex, primary nuclei ultimately break up into primary nuclei each containing one or two chromatin beads.

In the compound nucleus the primary nuclei most commonly occupy peripheral positions, or such positions that their chromatin granules most commonly lie at the periphery of the compound nucleus. The compound nucleus exhibits osmotic phenomena and its main body consists of a homogeneous fluid that stains red with methylene blue, the nucleoplasm. The nucleoplasm appears to have a cytoplasmic function and probably contains a RNA-metaphosphate complex. In the germinating spore, it becomes the cytoplasm of the germ cell. The compound nucleus divides by the formation of a membrane across the median part of the nucleus and subsequent splitting. The dividing membrane does not usually intersect any primary nucleus. On the other hand, the compound nucleus sometimes buds and the bud seems to contain two small beads of chromatin, each one apparently coming from a different primary nucleus. Accordingly, a bud after separation from the mother nucleus is fundamentally a small, compound nucleus.

This work confirms earlier observations by the reviewer (68) that nuclear fusion is extremely common in the bacteria. It also indicates that the extent to which fusion takes place is a function of certain influences tending to hinder growth. Since the association of primary nuclei into a compound nucleus usually precedes endospore formation and may be induced by certain toxic substances, it would appear that it is a natural process of increasing resistance. It must be added that in the complex nucleus the primary nuclei keep their identity and independence, and their association does not seem to be sufficiently intimate to justify designation in term of ploidy. The phenomenon of budding of compound nuclei may, in some instances, explain the configurations interpreted by the DeLamater school as mitotic stages with centrioles.

Birch-Andersen (2) developed a method of reconstructing nuclei from electron micrographs of serial sections. No new information has yet resulted. Kellenberger & Ryter (60) pointed out that the method of thin sections has given concordant results, but if the results are to be significant the technique involved has to be perfected.

Effects of various agents.—In cultures synchronized by exposure to cold temperatures, Szybalski & Hunter-Szybalska (144) observed a nuclear morphology resembling nuclei in metaphase. Parvis (112) studied the effect of "mitotic poisons" on the structure of *E. coli*, *Staphylococcus aureus*, and *Bacillus subtilis*. The effects seemed to be nonspecific and were observed when the substances were used in high concentrations. No figure that would correspond to any of the phases of mitosis was observed. Similar studies were carried out by DeLamater *et al.* (29), and by Minsavage & DeLamater (99, 100, 101). They concluded that many antibiotics and Na *p*-aminosalicylate, at inhibiting concentrations, induce an increase in the size and optical density of stained nuclei and a preponderance of configurations resembling the metaphase and anaphase stages. Continued chromosome reduplication results in a "transient polyploidy." Isoniazid and benzimidazole bring about a decrease in the amount of stainable material in the nucleus. Penicillin and bacitracin produce no change in the nuclear pattern. Colchicine produced four different responses and was effective in a wide range of concentrations.

The cellular division and the nuclear mechanism were inhibited in approximately 10 per cent of the cells. This resulted in what is interpreted as "polyploidy." In many instances the volume of cells was greatly increased.

It was shown by Schellenberg (134) that in the "large bodies" formed by *Proteus* in the presence of penicillin, the nuclear substance is mostly in the form of many small, round grains. Other large bodies may contain a mixture of fine and coarse grains or a network of nuclear material. Similarly, Olivo (110) had found that the giant cells of *E. coli* formed in media containing LiCl were multinuclear because of repeated division of the nucleus without division of the cytoplasm.

Kellenberger (58, 59) described a series of nuclear forms in *E. coli* and studied the effects of irradiation with ultraviolet light, x-rays, bacteriophage, and certain chemicals on nuclear morphology. The effects of Dichlorene are identical to those of ultraviolet light: at first the chromosomes increase in number, then they undergo fragmentation. Certain cells may recover and return to the original form, others may die. The sequence of events is similar in lysogenic and nonlysogenic cells. X-ray may cause three distinct effects, depending on the dosage. Weak doses cause an increase in the total number of "chromosomes," and even some fragmentation. Stronger doses induce the formation of vesicular nuclei. Very strong doses produce polar vacuoles. Induction results from fragmentation of the chromosomes and is caused by all inducing agents. Lieb *et al.* (93) found that nuclear and cell morphology are inherited as independent factors. Whitfield & Murray (161), employing

Shigella dysenteriae, and Maaloe *et al.* (94), with *E. coli*, confirmed earlier finding that the nuclear material of infected cells is usually present in the form of small granules. Whitfield & Murray described an early "condensation" of nuclear material followed by "expansion" into the granular form. Hartman *et al.* (51) and Payne *et al.* (113) described changes in cellular and nuclear morphology of lysogenic and nonlysogenic *E. coli* in response to irradiation with ultraviolet light. The changes were generally similar to those described by Kellenberger (see above). Clark & Webb (22, 23) pointed out difficulties in the way of drawing conclusions about ploidy from a study of irradiation death curves, but later attributed diploidy or polyploidy to the large cells of *Micrococcus pyogenes* var. *aureus* from a study of such curves. The survivors' curves of the large cells were sigmoid and those of the small cells exponential. The authors state that "Factors of clumping, multinuclearity, and multicellularity were investigated in order to validate ploidy interpretations." They also state that the nuclear structures observed in the large cells were similar to the reviewer's compound nuclei.

SLIME LAYER

Kaluzewski (57) noted that capsules may be demonstrated around coliform bacteria and staphylococci cultivated on an egg medium. The number of encapsulated cells is very high in cultures 2 to 4 hr. old and thereafter diminishes progressively. Tomcsik (148), and Tomcsik & Guex-Holzer (151) developed a method of demonstrating the capsule by means of a nonspecific, saltlike combination with protein. The reaction depends on the pH, and the pH depends on the isoelectric point of the protein used. Tomcsik (147) could not clearly observe "capsular transverse septa" (see 153) in *Bacillus anthracis* by his specific serological reaction until the cells had been incubated with the proper homologous serum for at least 17 min. Tomcsik & Grace (149) could not demonstrate these septa by any of several staining methods in several organisms studied.

Price & Kneeland (127) observed the capsular swelling phenomenon in a mucoid strain of *Micrococcus pyogenes* var. *aureus*. Labaw & Mosley (84) studied the structure of the capsule in a lysogenic strain of *E. coli*. They found striated fibers embedded in a homogeneous ground material. The striation had a periodicity varying from 200 to 290 Å, comparable to that of collagen fibers.

According to Morris & Seastone (104), trypsin, chymotrypsin, and papain increase phagocytosis of group A, β -hemolytic streptococci in the absence of specific antibodies, with no apparent effect on the structure of the capsule. This they interpreted as meaning that the M protein occupies a surface location on the capsule and, "either alone or in conjunction with hyaluronic acid, protects the streptococcus from the action of the leukocyte." Nordberg & Thorsell (109) studied the effect of certain enzyme systems on the capsule of *Bacillus anthracis*. This included enzyme preparations, extracts, or autolysates. The disintegration caused by lysozyme indicated the presence of

polysaccharide in the capsule. Autolysates of *B. anthracis*, *B. mesentericus*, *B. megaterium*, *B. subtilis*, *Streptococcus equi*, and *Streptococcus uberis* did not have any effect. On the other hand, autolysates from *Diplococcus pneumoniae* and *Serratia marcescens* had a disintegrating action.

Heidelberger & Adams (52) showed that various polysaccharides that contain glucose bound in positions 1, 4, and 6 (such as glycogen, amylopectin, and jellose), suitably bound glucuronic acid groups (e.g., gum arabic), or rhamnose (e.g., karaya gum, the high rotating polysaccharide of the tubercle bacillus and the C substance of group A, hemolytic streptococci) in their repeating units precipitate the antiserum of type II pneumococcus whose capsular polysaccharide consists of rhamnose, glucose linked in positions 1, 4, and 6, and glucuronic acid. Smithies & Gibbons (141) demonstrated a DNA slime layer around the cells of some halophilic bacteria. Treatment of the cells with deoxyribonuclease removed 50 per cent of the DNA without affecting viability. This report is of considerable interest to the reviewer who, in co-operation with Hillier & Fabricant (73) demonstrated slime possessing several of the properties of nucleic acids around the cells of an avian strain of *Mycobacterium tuberculosis*.

Duguid & Wilkinson (33) studied the influence of K deficiency upon the production of polysaccharide by *Aerobacter aerogenes*. Pierce & White (121) found galactose unsuitable for the synthesis of hyaluronic acid by *Streptococcus pyogenes*. Edmunds (34) added six more capsule types to the long list of such types in *Klebsiella*. The total now is 69. Knoll (75) demonstrated the importance of the capsule for the virulence of *Klebsiella pneumoniae*. Non-pathogenic doses become pathogenic if mucin or capsular substance is injected simultaneously. In a similar manner a nonpathogenic strain may become able to cause disease, but this property is not inherited. The capsule substance simply blocks phagocytosis.

CELL WALL

Wigand *et al.* (164) found a cell wall around *Bartonella bacilliformis*. Tomcsik & Guex-Holzer (152) made use of the specific reaction of the cell wall with its homologous antibodies to demonstrate the cell walls and cross walls of a *Bacillus* of the cereus-megaterium group with the electron microscope. They also used a nonspecific reaction with protein at appropriate pH for a similar purpose. Both procedures had been used by the authors in the study of the capsule (150, 151). The results led to the conclusion that the D-glutamic acid polypeptide demonstrated in the capsule does not occur in the cell wall. Study of the serological effects of cell wall preparations from which the protoplasm was removed by treatment with trypsin showed that the cell wall is responsible for most of the serological reactions of the cell surface. Naked protoplasts, freed of their cell walls by the action of lysozyme induced the formation of antibodies that reacted with thermolabile protein of the cell surface.

The large, spherical cells produced by *B. anthracis* in the presence of

penicillin are surrounded by cell walls (148), a fact that has also been demonstrated by others (142, 146) in *Proteus*. Cell walls of strains of *Bacillus*, *Micrococcus*, and *Caryophanon* may be stained with Alcian blue and show a structure similar to that shown by the specific serological reaction when observation is made with the phase microscope (149). Girbardt & Taubeneck (40) showed that the method of Chance (16) does not actually stain the cell wall but forms a flocculation membrane on the cell wall. Yoshida *et al.* (168) stained the cell wall, and the cell wall and cross wall of *B. subtilis*, for observation with the light microscope using, respectively, oronine and Congo red and oronine and Victoria blue. They also observed the cytoplasmic membrane with the electron microscope. Neither Morton *et al.* (105), nor Cuckow & Klieneberger-Nobel (24) could demonstrate, with the electron microscope, a cell wall in the pleuropneumonia group.

Labaw & Mosley (85, 86) observed a rectangular array, with a periodicity in each direction of about 115 Å, on the inside surface of a gram negative bacillus. The outside surface showed no structure. The thickness of the cell wall was about 75 Å, a low value when compared with measurements made by others on other bacteria. Salton & Williams (132) observed a macromolecular structure in the cell wall of *Rhodospirillum rubrum* but not in those of *Pseudomonas fluorescens*, *Rhodopseudomonas spheroides*, *E. coli*, and *B. megaterium*. It should be recalled that Knaysi & Hillier (72) observed a fine structure in the wall of the latter organism. Indeed, Figure 2 published by Salton & Williams shows that cell walls of *B. megaterium* dried from the frozen state did exhibit a macromolecular structure, although of a type different from that observed in *R. rubrum* as illustrated in their Figure 4. On the other hand, the cell wall of the *Rhodospirillum* did not show any structure when dried from the frozen state, nor did a batch apparently similar to those that showed structure. A priori, all cell walls may be considered as possessing a macromolecular structure. Whether or not this structure is seen would depend on whether or not it is masked by other material with which it is associated. The reviewer has always visualized the cell wall as consisting of coarse macromolecules embedded in a much finer ground material that appears homogeneous with the electron microscope. This concept is in harmony with the observations of Knaysi & Hillier (72) made on cell walls and spore coats of *B. megaterium*. In the case of the spore coat, it was noted that structure became clearly visible only in coats that were for a while under conditions favoring autolysis. In freshly shed coats there was no observable structure.

Houwink (53) explained his observations by assuming that the cell wall of his *Spirillum* consists of two or three superposed layers. This also appears to be a possible explanation in the case of the gram negative bacillus of Labaw & Mosley (see above), although it is hard to conceive that a cell wall of 75 Å is made up of more than one layer. Such layers of biological material of low density would probably not be visible with the electron microscope. Study of the structure of the cell wall is still in its infancy, but it is an important subject. Macromolecular structures of different organisms may show

differences of significance related to the properties and behavior of these organisms. Few (35) observed a "secondary layer or membrane" within the outer cell walls derived from *Pseudomonas denitrificans* and *E. coli*, but not in cell walls derived from *Micrococcus pyogenes* var. *aureus*. The first two strains were sensitive to polymyxin E, and treatment of their free cell walls with that substance resulted in marked morphological changes. Cell walls of the resistant staphylococcus did not show any significant changes.

Yoshida *et al.* (167) noted that, when observed with the electron microscope, the outer surface of the cell wall of *B. subtilis* appeared rough after treatment with 0.1 per cent phosphotungstic acid, in contrast with the inner surface which appeared smooth. However, both surfaces appeared smooth when the cell walls were treated with pepsin and trypsin before treatment with phosphotungstic acid, indicating the presence of a protein component in the outer layer of the cell wall. Boiling of the free cell walls of *E. coli* B (166) with distilled water or *N* HCl gave rise to bundles of filaments. The thickness of a filament was 40 to 50 μ . Boiling with 0.01*N* NaOH gave cell wall skeletons with transverse structural orientation. The cell wall of *Salmonella typhosa* (165) broke up in boiling distilled water into discs connected by filaments. In boiling 0.01*N* NaOH the cell wall became granular; in boiling *N* HCl it gave rise to fine filaments, 20 to 30 μ in thickness, which changed in 60 min. to granular material that disappeared upon longer exposure. Appearance of the cell wall when treated with phosphotungstic acid after exposure to a homologous serum suggested that the antigens are arranged in a mosaic.

Grula & Hartsell (44, 45) studied the action of lysozyme on the cell wall of *Micrococcus lysodeikticus*. They confirmed previous observations by others that lysozyme destroys the cell wall. In double distilled water, destruction of the cell wall is immediately followed by disruption of the protoplasm, but in a medium of osmotic pressure similar to that of 0.85 per cent NaCl "ghosts" (?) are formed. "Dense and highly opaque intracellular granules are not destroyed by lysozyme, although deoxyribonucleic acid is liberated from the cell. . . . Alcian blue is an excellent cell wall stain for this organism," and when cell wall preparations are saturated with the dye, complete inhibition of lysis takes place. Solubilization of the cytoplasm by alkali (the Nakamura effect) was possible only after the cell wall was sufficiently degraded by lysozyme to permit contact between the alkali and the cytoplasm. The change in the Gram reaction of the cells treated with lysozyme is attributable to an increase of permeability caused by the removal of the lysozyme substrate. Free cell walls are gram negative. More recently, Grula & Hartsell (46) showed that gram negative bacteria also may be affected by lysozyme under proper conditions of pH, temperature, age of culture, and use of the Nakamura effect. The action was demonstrated on whole cells and on isolated cell walls. Of 22 species (representing 11 genera) tested, *Neisseria perflava*, whose wall stained well with Alcian blue, behaved as *M. lysodeikticus*, i.e., its cell wall was lysed even at pH 7. The cell wall of *K. pneumoniae* also

stained fairly well with Alcian blue, but lysis was incomplete. The cell of *Vibrio comma* was disrupted by alkali without the addition of lysozyme. Warren *et al.* (158) reported lysis of *Pseudomonas aeruginosa* and "other gram negative bacteria" by lysozyme without resort to the Nakamura effect. The cells were pretreated with heat or acetone. "Evidence was presented . . . that an autolytic system influenced the action of lysozyme. . . . The optimum pH for lysis of acetone-treated suspensions . . . was . . . between 7 and 8."

Chemical analysis of the cell wall of *R. rubrum* by Salton & Williams (132) gave 55 per cent protein, 23 per cent reducing substances liberated by hydrolysis with 2 N HCl, and 21 per cent lipide. Aromatic amino acids were present. Bowen (9) made electrophoretic and diffusion measurements on cell wall oligosaccharides of *Corynebacterium diphtheriae*. The saccharides had zero mobility and a diffusion coefficient of 2.82×10^{-6} cm./sec., corresponding to a molecular weight of about 1220. Tomcsik (147) noted differences among members of *Bacillus* in the resistance of their cell walls to lysozyme. *B. megaterium* was highly susceptible, whereas *B. cereus* and *B. anthracis* were highly resistant. Cummins & Harris (25) found a specific protein antigen and a group antigen, apparently a polysaccharide, in the cell wall of *C. diphtheriae*. The specific antigen is hydrolyzed by pepsin into a number of amino acids. The group antigen is inactivated by 0.01 M periodate and contains arabinose, galactose, and mannose. Barkulis & Ekstedt (1) analyzed cell walls of group A, hemolytic streptococci (type 14). On the basis of dry weight, the cell walls amounted to 25 per cent of the whole cells, which agrees well with the purely morphological estimate made by the reviewer (67). C polysaccharide, which consists of rhamnose and hexosamine, made up 30 to 35 per cent of the cell walls. The remaining 65 to 70 per cent consisted of protein, it contained 9.5 to 10 per cent of nitrogen and the major portion of the M antigen. These authors do not believe that nucleic acid is a structural component of the cell wall.

FLAGELLA

Value in taxonomy.—Several years ago, the reviewer (66, 67) pointed out that the taxonomic use of flagella is empirical and does not depend on their origin or function. Peluffo (114), who for over 10 years had been preparing "pure flagellar suspensions," reported a remarkable constancy in the "spiral period" of a given strain of bacteria. His observations with the phase microscope furthermore indicate that certain groups of bacteria may be distinguished from one another by the spiral period of their flagella. Monotrichous organisms generally have a shorter period (1 to 1.70 μ) than peritrichous ones (1.95 to 2.50 μ). *Proteus morganii*, of doubtful systematic position, has a longer period (2.30 μ) than other species of *Proteus* (1.95 μ). *V. comma* has a longer period than nonpathogenic vibrios (150 μ). *B. subtilis* has a shorter period (2.15 μ) than *Bacillus brevis* (2.50 μ). Leifson (88) noted that within the genus *Acetobacter* atrichous and peritrichous species oxidize acetic

and lactic acids to CO_2 and water, whereas species with terminal flagella are unable to do so. Accordingly, he proposed division of the genus into two genera, *Acetobacter* and *Acetomonas*. Leifson & Hugh (90) founded a new species, *Pseudomonas diminuta* chiefly on the "wavelength" of its flagella (0.62μ). Pijper & Abraham (124) noted that in several motile bacteria, excluding *Spirillum*, one may find in the same culture, and indeed in the same cell, flagella of two different wavelengths, one being twice as long as the other. Thus in *Sarcina ureae* as well as in *Sarcina agilis* one finds wavelengths of 1.6 and 3.2μ . A similar relation was found in illustrations published by other investigators. Similar observations were made by Leifson *et al.* (89) on 60 strains of *Proteus*. The flagella showed four types of curvature: normal, curly, semicoiled, and coiled "The normal and curly are most common and basic. Individual organisms may have more than one type of flagella, and individual flagella may have one or two types of curves. The type of curvature varies with the strain." The mean wavelength of the normal flagella was 2.3μ and that of curly flagella 1.1μ . Environmental factors, particularly pH, may change flagellar curvature in certain strains. The normal and curly flagella of these authors correspond, respectively, to the flagella of long and short wavelengths of Pijper & Abraham, and the greater variation found by Leifson *et al.*, familiar to observers of stained flagella, may be an artifact of drying, fixing, and staining. The anaerobic genus *Selenomonas* has been recognized by its peculiar type of flagellation (92). The flagella of *Listeria* were studied by Leifson & Palen (91). Goldin & Menolasino (42) found a correlation between somatic antigenicity and type of flagellation in *Alcaligenes*. Menolasino *et al.* (97) noted a change in flagellar morphology when an organism is grown in a medium containing the homologous anti H serum.

Structure, form, dimensions, and origin.—Labaw & Mosley (84, 85, 86) studied the structure of the flagella in a gram positive, nonsporulating organism and in *Brucella bronchiseptica*. They concluded that flagella have the external contour of a counterclockwise, triple helix. In the case of the *Brucella*, the average periodicity was 190 \AA , the average width 139 \AA , and the average slope 54.5° . Grace (43) noted that in most spirilla there are from 10 to 30 flagellar fibers that emerge from the cell in bundles. The average diameter of a fiber is $10 \text{ m}\mu$ compared with 20 to $28 \text{ m}\mu$ in *Vibrio* and *Pseudomonas*. *R. rubrum* had fewer flagella that, in ghost cells, appeared to arise from a single basal granule. Ghost cells were produced by incubation in distilled water for 18 hr. at 5°C . In *V. comma* the cells had each a single, stout, polar flagellum that arose from a basal granule $100 \text{ m}\mu$ in diameter. Grace concluded that in *Spirillum* the flagellar apparatus is intermediate between that of the flagellates and that of other bacteria. In the latter, according to the work of others, each flagellum arises from a separate blepharoplast. Pijper *et al.* (125) found in sarcinae the same type of flagella as in *S. typhosa* and other peritrichous organisms. Pijper (122) reaffirmed his belief that there are two types of flagella. In *Spirillum*, the flagella are "horny-looking," curly, and are directly derived from the cell wall. Other bacteria, however,

usually go forward with a fuzzy-looking straight tail . . . and a twisting body. Under various, probably adverse, conditions this straight tail tends to stiffen into a straight, clear-cut rod but more often into a clear-cut helix. . . . The helices may show two different but characteristic wave-lengths in the same bacterium, one always exactly twice the other . . . for which phenomenon "biplicity" would appear a suitable term.

The change from straight tail to helix is not accompanied by a reduction in the apparent length (i.e., the rectilinear distance from tip to base), which means that the change is accompanied by an increase in the real length of the tail. The relative increase is variable but may amount to 50 per cent. The process, at first reversible and not affecting motion, "may well create tensions or liberate forces capable of setting up . . . anomalous transient whirling. . . ." This is apparently an explanation by Pijper of the whirling movement of carmine particles in microcultures of *Caryophanon* used by others (96) as a proof that flagella are motor organs. More recently, Pijper (123) studied two new spirilla and observed polar and lateral flagella. He concluded that the

Cell walls of spirilla apparently contain a network of submicroscopic fibrils. These may become twisted into typical and atypical flagella, and may also come off as masses of fibrils. None of these structures can be regarded as motor organs.

MacDonald *et al.* (95) observed in dark field five strains of *Fusobacterium girans*, an organism previously described by Prévot under the name of *Fusobacillus girans*. They observed three types of movement: a sudden lashing around a fixed pole as if one end of the cell were fixed to the slide or coverslip; a flexing of the cell; and a gliding progression through the suspending medium at rates up to 5 μ per second. The latter type was seen only in relatively short cells up to 10 μ in length. No flagella were demonstrable directly or indirectly in dark field, nor with the electron microscope. There was evidence for the presence of a slime layer. Motility was not affected by the addition to the medium of surface-active agents, but was inhibited by homologous serum. Prévot *et al.* (126) also could not find flagella, but in shadowed preparations demonstrated with the electron microscope what they described as enormous, reticulate, mucilaginous expansion in subpolar position. This they considered as a new type of motor organ.

Function.—By means of the electron microscope, Smith (140) demonstrated flagella in eleven motile strains of *Aerobacter cloacae* and nine motile strains of *E. coli*. No flagella were demonstrable in a nonmotile strain of the first organism or in seven nonmotile strains of the second. This was considered evidence that flagella are organs of locomotion. Cultures of *E. coli* in synthetic, liquid media had "immobile flagella," and the addition of phenol to the medium resulted in the loss of flagella and motility. Reference has already been made to the observation of Pijper *et al.* (125) on sarcinae and to those of Pijper (123) on two new spirilla. In neither case does he consider flagella as motor organs: motility in sarcinae is a result of continuous tumbling of the packets, caused by intracellular activities; it is not likely that these

structureless fibers could generate the necessary energy for the observed motion; the packets are covered by jelly-like material which can be drawn into a tail and allows unrestricted tumbling inside it; stiffening of the slime stops tumbling; stiffening of the tail is accompanied by increase in real length, which may result in flagellar motion. Pijper's conclusions about spirilla have been summarized above.

Kvittingen (83) noted that *Proteus* cells in the swarming stage are highly flexuous and suggested that some of the cell movements, which were recorded by cinematography, are attributable to active movement of the cell body. Flagella-like appendages were frequently still attached to cells in disintegration. Morowitz (103) calculated that the energy necessary for the motility of a single cell of *B. subtilis* is about 56 ev per sec. and concluded that "it is possible to consider each flagellar flick as the result of a small number of discrete chemical events (perhaps one), such as metabolic hydrolysis of energy rich phosphate bonds." However, these calculations took into consideration the translational movement only.

Comment.—It is the considered opinion of the reviewer that the status of knowledge regarding the origin and function of flagella is still in a chaotic condition. Different investigators using similar or different methods draw different conclusions. Flagella attached to ghost cells appear to indicate a peripheral origin of the flagella. Flagella still attached to lysozyme-treated cells (160) appear to indicate a protoplasmic origin of the flagella. This situation is reminiscent of the one which, until recently, prevailed about the nucleus. It clearly indicates that there is something definitely wrong with present knowledge, not only about flagella but also about other peripheral structures of the cell.

The work of the last two years showed that there may be more than one type of appendages around swimming bacteria. Certainly, the flagella illustrated by Grace and the appendages illustrated by Prévot *et al.* do not appear to be of the same type. The formation of flagella by immobile cells (140) is not new (cf. 63), and may be supplemented by a heretofore unreported observation by the reviewer on giant flagella in *E. coli* and a gram negative *Bacillus* resembling *B. brevis* with the phase microscope: cells that carried giant flagella were invariably immobile. The giant flagella were so rigid that they swayed, but did not bend or undergo any other change in form or structure, when squarely hit by actively motile cells. Since the life history of the cells which carried the giant flagella is not known, immobility may be due to rigidity of the giant flagella, or rigidity of the flagella may be accompanied by rigidity of the cell wall which apparently does have some flagellar antigen. Immobility of cells carrying normal flagella is also subject to these two alternate explanations. It is extremely difficult to prove that an immobile, flagellated cell did not undergo any type of motion at some period prior to observation. The strongest evidence for the formation of flagella without previous motion was reported by Knaysi & Hillier (67) but even here twisting of the cell without displacement can not be entirely ruled out.

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METABOLISM OF CARBOHYDRATES AND RELATED COMPOUNDS^{1,2,3}

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This year the Editorial Board has divided the heroic task of reviewing microbial metabolism into two parts; this division simplifies the task of the reviewer to a considerable extent, but still leaves many aspects of a large area to be covered. This review has tried to cover the journals received in the year 1955. Abstracts of papers delivered at meetings are not thoroughly reviewed since they are already available in concentrated form, but many have been included if they seemed to fit into a subject being developed.

Numerous reviews and symposia appear each year on aspects of the metabolism of microorganisms, and the subject has been reviewed each year in this series, so the main role of the Annual Review appears to be that of assembling the publications of the year with some indication of the status of the subject. The variety of papers reported on should indicate the inability of a single reviewer to comment adequately on their significance and relationships.

The year has seen the publication of many of the experimental details which pertain to the study of the enzymes and enzyme systems concerned with the various pathways of carbohydrate metabolism about which so much has been said at meetings and symposia. The assessment of the role of these pathways in the metabolism of various microorganisms has been the subject of many papers. The tricarboxylic acid cycle continues to provoke experiment and comment. The variety of microorganisms, growth conditions, and substrates make plain the amount of work that remains to be done.

The following reviews have appeared: "Mechanism of action and properties of pyridine nucleotide-linked enzymes" by Racker (1), "Significance of alternate pathways in the metabolism of glucose" by Wood (2), "The

¹ The survey of the literature for this review was completed in December, 1955

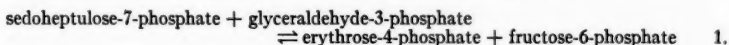
² The following abbreviations are used: acetyl CoA for acetyl coenzyme A; ADP for adenosinediphosphate; AMP for adenosinemonophosphate; ATP for adenosine-triphosphate; CoA for coenzyme A; DPN for diphosphopyridine nucleotide; DPNH for reduced diphosphopyridine nucleotide; DPT for diphosphothiamine; IDP for inosinediphosphate; ITP for inosinetriphosphate; P for inorganic phosphate; PP for pyrophosphate; TPN for triphosphopyridine nucleotide; TPNH for reduced triphosphopyridine nucleotide.

³ This review was prepared while the author was associated with the Microbiology Unit, Department of Biochemistry, University of Oxford during the tenure of a fellowship from the John Simon Guggenheim Memorial Foundation and the Fulbright Commission. The author wishes to thank Professor D. D. Woods for his hospitality and interest.

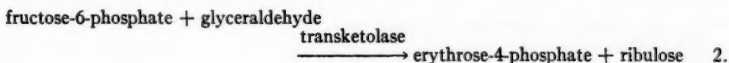
enzymatic degradation of polysaccharides" by Manners (3), " α -Lipoic acid," and "Metalloflavoprotein enzymes" by Long (4, 5), and "Pathways of carbohydrate metabolism in microorganisms" by Gunsalus, Horecker & Wood (6). The availability of the latter review, which appeared in the middle of the year, has permitted the present reviewer to be sparing of explanation and diagram in the areas covered by this review.

ENZYMES

The glucose-6-phosphate dehydrogenase of brewer's yeast was purified by Glaser & Brown (7) who have reported its properties; the glucose-6-phosphate dehydrogenase of *Bacillus subtilis* studied by Marquet & De-donder (8) seems to have similar properties. Transaldolase has been purified 400-fold from yeast by Horecker & Smyrniotis (9). This enzyme, for which cofactors have not been observed, catalyses the reaction:



The erythrose-4-phosphate was identified by Horecker *et al.* (10) by its reaction with dihydroxyacetone phosphate to form sedoheptulose-1,7-diphosphate in the presence of muscle aldolase, and by its reaction with active glycolaldehyde from the transketolase system to yield fructose-6-phosphate. Kornberg & Racker (11) isolated erythrose-4-phosphate as a product of the reaction



and compared its behavior in several enzymatic reactions, in Dische's colorimetric reaction, and on paper chromatography with that of synthetic erythrose-4-phosphate provided by Ballou, Fischer & MacDonald (12). The compounds were identical as indicated by their behavior in the reactions studied.

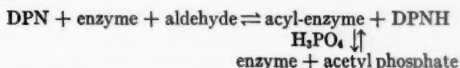
Transketolase, crystallized from yeast by de la Haba, Leder & Racker (13), apparently used ribulose-5-phosphate as a substrate and transferred an active glycolaldehyde group to an appropriate acceptor. The products were glyceraldehyde-3-phosphate and a keto sugar, whose nature depended on the acceptor. Diphosphothiamine and magnesium ions functioned as cofactors.

Dickens & Williamson (14) have shown that a Walden inversion converted ribulose-5-phosphate to xylulose-5-phosphate. That xylulose-5-phosphate is the substrate of transketolase was demonstrated by Sreere *et al.* (15). The "ribulose-5-phosphate" used by de la Haba *et al.* (13) was prepared by the action of pentose phosphate isomerase of yeast and was actually a mixture of ribose-5-phosphate, ribulose-5-phosphate, and xylulose-5-phosphate.

Kovachevich & Wood (16) reported the isolation and properties of the

aldolase from *Pseudomonas fluorescens* that converts 2-keto-3-deoxy-6-phosphogluconate to pyruvate and glyceraldehyde-3-phosphate. No cofactors have been demonstrated. Sulfhydryl inhibitors and metal chelators have no effect on the enzyme. The enzyme is found in several gram negative organisms, but not in gram positive ones. The 2-keto-3-deoxy-6-phosphogluconate was shown to be formed from 6-phosphogluconate by another enzyme of *P. fluorescens*, called 6-phosphogluconate dehydrase, which was separated from the aldolase by Kovachevich & Wood (17). Ferrous ions and glutathione were required for maximal activity.

Krimsky & Racker (18) found that yeast glyceraldehyde-3-phosphate dehydrogenase reacted with acetyl phosphate or 1,3-diphosphoglycerate to form acyl compounds stable in the absence of DPN. These thiol ethers could be reduced to yield aldehydes by DPNH. This confirms the mechanism previously suggested:



The mechanism of this reaction is established now by the isolation of an intermediate after years of experiment and speculation. Halsey (19) has shown that the thiol groups of yeast glyceraldehyde-3-phosphate dehydrogenase were differentially attacked by methyl mercury nitrate since the addition of two moles of mercury compound per mole of enzyme caused only slight reduction of activity; four moles were required for complete inhibition. On the other hand two moles of *p*-chloromercuribenzoate induced complete inhibition. Thus the two thiol groups that react readily with methyl mercury nitrate are not required for the activity of the enzyme.

An iron-activated aldolase has been detected in extracts of *Brucella suis* by Gary, Kupferberg & Graf (20). A xylose isomerase, which catalyses the formation of a mixture of 16 per cent xylulose and 84 per cent xylose, has been found in extracts of *Pasteurella pestis* by Slein (21). The xylose isomerase of *Pseudomonas hydrophila* has been shown by Hochster (22) to be inhibited by various sulfhydryl reagents, but only *p*-chloromercuribenzoate induced inhibition was reversed by glutathione. Sonic extracts of *P. hydrophila* were found by Hochster (23) to convert D-xylose to D-xylulose and D-xylulose-5-phosphate, which was further metabolized; D-xylose-5-phosphate was not. The phosphoglucomutase and the phosphoribomutase of yeast and mammals have been shown to be distinct enzymes by Guarino & Sable (24). That *Aspergillus niger* contains an isocitric dehydrogenase, similar to that of yeast and animals, has been shown by Ramakrishnan & Martin (25). Dolin (26, 27) has studied several flavoproteins of *Streptococcus faecalis* that react with various oxidants. The DPNH-peroxidase has been purified 100-fold. Woody & Lindstrom (28) have purified the succinic dehydrogenase of *Rhodospirillum rubrum*. The particulate fraction they obtained oxidized succinate to fumarate in the presence of 2,6-dichlorophenolindophenol or cytochrome-*c*.

A mechanism for the action of the citrate splitting enzyme of *Aerobacter aerogenes* has been suggested by Dagley & Dawes (29) which accounts for the proportionality of enzyme action and enzyme concentration, the powerful inhibition by oxalacetate, and the activation by magnesium, zinc, or ferrous ions. A chelate type compound of metal, enzyme protein, and citrate is split to yield acetate and oxalacetate-metal-enzyme which does not easily regenerate active enzyme. The formation and distribution of isocitratase has been reported by Smith & Gunsalus (30, 31). Aerobic conditions were required for production of the magnesium ion and mercapto compound-activated enzyme, found in *Pseudomonas*, *Azotobacter*, *Acetobacter aceti*, *Escherichia coli*, and *Serratia marcescens*. The enzyme is not present in *Micrococcus lysodeikticus*. The lactic acid oxidative decarboxylase of *Mycobacterium phlei* was isolated in an electrophoretically pure state by Sutton (32) and found to contain a flavin prosthetic group that was similar, but not identical to riboflavin-5'-phosphate. Brodie & Lipmann (33) have shown that a lactonase was required to open the ring of the δ -lactone formed when glucose was oxidized. The enzyme is found in a variety of tissues and when purified 36-fold required divalent ions for activity. The 6-phosphogluconolactone is also hydrolyzed, as indeed are a variety of lactones with the same configuration as D-glucono- δ -lactone. Valee & Hoch (34, 35) found a high zinc content (four Zn per mole enzyme) of purified yeast alcohol dehydrogenase. They suggested that four moles of DPN were bound; but in the liver enzyme, with only two atoms of Zn per mole of enzyme, only two moles of DPN were bound.

Yeast alcohol dehydrogenase has been found by Kuff, Hogeboom & Striebich (36) to sediment as a single component from crude dilute solutions and from solutions of pure enzyme. The removal of thioctic (lipoic) acid from cell-free extracts of *Tetrahymena pyriformis* and *S. faecalis* by alumina treatment has been reported by Seaman & Naschke (37). The pyruvate oxidase of pigeon muscle and the α -ketoglutarate oxidase of pig heart required the addition of a pigeon liver fraction before alumina would adsorb lipoic acid. Presumably the pigeon liver fraction freed the lipoic acid for adsorption.

CARBOHYDRATE CATABOLISM

Fermentation mechanisms.—The chief method used for investigation of the route of fermentation was the determination of the location of tracer atoms in the products produced by the fermentation of labeled substrates. Altermatt, Simpson & Neish (38) used glucose-1-C¹⁴ and glucose-2-C¹⁴ as fermentation substrates for *Aerobacter aerogenes* and found the results compatible with the classical Embden-Meyerhof scheme; that is the glucose-1-C¹⁴ yielded products with labeled methyl groups and the glucose-2-C¹⁴ yielded products with labeled carbinol groups, or carboxyl group in the case of acetic acid. D-Allose was fermented to the same products with the same label distribution as D-glucose, although they differ in the position of the

hydroxyl group on carbon 3. The exposure of thin layers of the reaction mixture to air produced little change in the amount or distribution of the products, which led the authors to conclude that the hexosemonophosphate pathway played a small role aerobically. However, the failure to reduce the amount of fermentation products suggests that the conditions were not actually aerobic, and judgment should be withheld until more positive methods of producing aerobic conditions within the suspension have been tested.

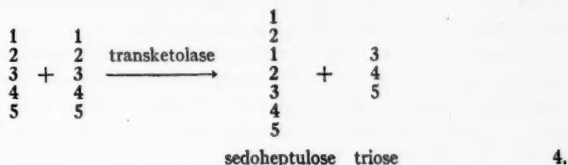
The fermentation of D-ribose-1-C¹⁴, D-xylose-1-C¹⁴, D-xylose-2-C¹⁴, and D-xylose-5-C¹⁴ by *A. aerogenes* provided further evidence that the fermentation of pentoses by the Enterobacteriaceae differs from that of the Lactobacteriaceae. In the latter essentially a 3-2 split of the pentose molecule occurs. In the former, the labeling of the products indicates that the pentose is converted to 3-carbon fragments by way of 6- and 7-carbon intermediates (presumably fructose-6-phosphate and sedoheptulose-7-phosphate) [Altermatt, Simpson & Neish (39)]. Sedoheptulose was also fermented by *A. aerogenes* to yield the same products as obtained from glucose, but analysis of the distribution of the label in the products of the fermentation of sedoheptulose-2-C¹⁴ and sedoheptulose-1-C¹⁴ indicates that, in addition to the expected hexosemonophosphate and the Embden-Meyerhof pathways, other reactions, such as carbon dioxide fixation, must also have occurred [Neish & Blackwood (40)].

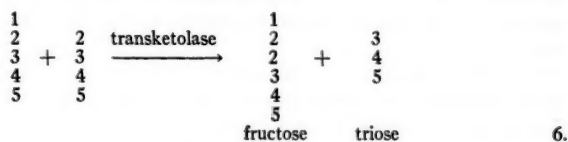
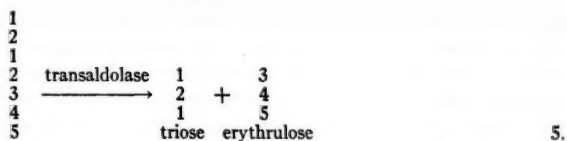
Cell-free extracts of yeast fermented ribose-1-C¹⁴ in the same manner as postulated for *A. aerogenes*, yielding ethanol with approximately twice the concentration of label in the methyl group as in the carbon dioxide. [Gibbs (41); Gibbs, Earl & Ritchie (42)].

Cell suspensions of *E. coli* fermented D-xylose-1-C¹⁴ to lactate, acetate, and formate and smaller amounts of succinate and carbon dioxide [Paegle, Gibbs, & Ritchie (43)]. The methyl groups of lactate and acetate had the greatest activity and the carbon dioxide and carboxyl groups of lactate, succinate, and formate had 0.6 to 0.7 of the activity of the methyl groups. The carbinol carbon of the lactate, and the carboxyl carbon of acetate were inactive.

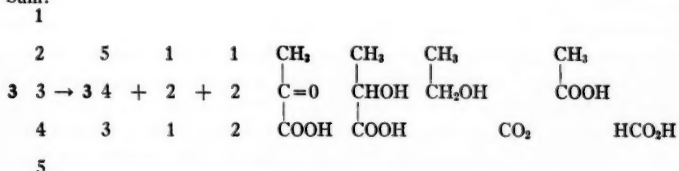
Extracts of acetone powders of *E. coli* converted ribose-5-phosphate-1-C¹⁴ into hexosemonophosphate labeled in the 1- and 3- positions, but carbon 1 had 2.4 times the activity of carbon 3 [Gibbs, Paegle & Earl (44)].

These pentose fermentations may be diagrammed as follows, the numbers representing the carbon atoms:





Sum:



Altermatt, Blackwood & Neish (45) have determined the products produced by *Leuconostoc mesenteroides* from D-xylose-1-C¹⁴, D-xylose-2-C¹⁴, and D-xylose-5-C¹⁴ and found that distribution of the label indicated the 3-2 split type of fermentation already known in some lactobacilli. Bernstein and co-authors (46) have demonstrated that the fermentation of glucose by *L. mesenteroides* may be used to obtain each carbon of the glucose as a separate fraction with very little cross contamination; thus this method is superior to the widely used *Lactobacillus casei* fermentation which yields only pairs of the glucose carbons, i.e., 1-6; 2-5; 3-4. A study of several lactic acid bacteria confirmed the expectation that homofermentative types fermented glucose-1-C¹⁴ in the same manner as *L. casei* and that the heterofermentative types fermented glucose in the same manner as *L. mesenteroides*. But the occurrence of inducible pentose and aldonic acid fermentations by a different route in the homofermentative types indicates the versatility of the micro-organisms [Gibbs, Sokatch & Gunsalus (47)].

Evidence for an Embden-Meyerhof pathway and a hexose monophosphate-pentose pathway in *Microbacterium lacticum* was reported by Krichewsky, Van Demark & Wood (48). However, ribulose-5-phosphate-2,3-C¹⁴ was converted to carboxyl-labeled pyruvate, and ribose-5-phosphate-1-C¹⁴

was converted to unlabeled pyruvate by cell-free extracts. These data indicate that this species cleaved pentose in the same general fashion as the other Lactobacteriaceae. Green & Cohen (49) have demonstrated the conversion of the methyl pentose L-fucose to L-fuculose by cell-free extracts of D-arabinose-grown *E. coli*.

The propionic acid fermentation has been studied further by Wood, Stjernholm & Leaver (50) using glucose labeled in the 1, 2, 6, or 3 and 4 positions and two species of *Propionibacterium*. As was expected from previous work, the intermediate reactions were complex and labeled carbon appeared to be distributed haphazardly. Much of the carbon dioxide came from the 3- and 4-carbons, but considerable amounts were produced from the 1-position, and much less from the 2- or the 6-position; this indicated that the Embden-Meyerhof route was a major, but not the sole, path. Fermentation by the two species varied in some respects and one species sometimes produced propionate with a higher degree of labeling than succinate which indicated that succinate was not the sole precursor of propionate. A comparison of the fermentation of three-carbon substrates by *Clostridium propionicum* and *Propionibacterium* was made. The *Clostridium* produced propionate-3-C¹⁴ and acetate-2-C¹⁴ from lactate-3-C¹⁴, suggesting a direct reduction. The propionic acid bacterium, however, yielded propionate labeled in the 2- and 3-positions chiefly, but the label also appeared in the carboxyl group and in the carbon dioxide produced. Both carbons of the acetate produced were labeled, but the methyl was labeled to a greater extent than the carboxyl carbon. Compatible results were obtained using carbon-2 labeled lactate, pyruvate, or glycerol. The authors suggested that succinate is the precursor of propionate, and that citrate might be an intermediate [Leaver, Wood & Stjernholm (51)].

In a series of papers, Milhaud & Aubert (52, 53) and Aubert & Milhaud (54, 55) have reported the products obtained following utilization of radioactive glucose or ethanol by baker's yeast. They employed chromatography of the reaction mixture to separate and identify the radioactive products. The authors demonstrated that labeled ethanol is converted into all the products of usual fermentation. The distribution of label in the trehalose produced indicated formation by way of the Embden-Meyerhof scheme from two three-carbon fragments. This was apparently synthesized in two ways: through the citric acid cycle or by carboxylation of the two-carbon fragment to a three-carbon compound.

Marmur & Hotchkiss (56) have studied the transfer of an inducible ability of pneumococci to ferment mannitol. A highly polymerized deoxyribose nucleic acid from a mannitol-fermenting mutant converted cells incapable of using mannitol into cells which could be induced to ferment mannitol following the addition of mannitol and a complex nitrogen source. The fermentation is postulated to occur by the phosphorylation of mannitol to mannitol phosphate, although this reaction has not been demonstrated.

The oxidation of mannitol phosphate to fructose-6-phosphate by a new DPN-linked dehydrogenase was studied in some detail.

The synthesis of acetate from formate and carbon dioxide by *Clostridium thermoaceticum* using C^{14} -labeled substrates has been reported by Lentz & Wood (57). $C^{14}O_2$ is converted to totally labeled acetate in short term experiments. Carbon dioxide and formate were rapidly equilibrated but formate was a better precursor of the methyl group of acetate, and carbon dioxide was a better precursor of the carboxyl group of acetate. Formaldehyde was reduced to formate.

Another fermentative pseudomonad was reported by Crawford (58). The fermentation pattern resembles that of an aerobically-grown *E. coli* which cannot break down formate. Since formate predominates in the fermentation products the name *Pseudomonas formicans* n. sp. was suggested.

Mantoya & Bolcato (59) have isolated the 2,4-dinitrophenyl hydrazide of butraldehyde from *Clostridium acetobutylicum* fermentations, and have suggested that butraldehyde is an intermediate in the reduction of butyric acid to butanol. *L. mesenteriodes* extracts were found to oxidize the phosphate ester of 2-deoxy-D-glucose by a TPN-linked dehydrogenase partially separable from glucose-6-phosphate dehydrogenase [De Moss & Happel (60)]. No inhibition of glucose-6-phosphate dehydrogenation by the sugar or its phosphate ester was obtained.

Blakley & Boyer (61) have shown that 6-deoxy-6-fluoro-D-glucose inhibits fermentation by yeast cells when present in concentrations of the order of the sugar concentration. The compound had no inhibitory effect on fermentation by cell-free extracts or on hexokinase. Since the inhibitory effect increased as the sugar concentration was lowered it was concluded that the entrance of sugar into the cell was effected.

Oxidation mechanisms.—Two routes of formation of dihydroxyacetone phosphate by *Acetobacter suboxydans* have been demonstrated by Hauge, King & Cheldelin (62, 63). Glycerol may be oxidized to dihydroxyacetone and then phosphorylated, or it may be phosphorylated to α -glycerophosphate which is then oxidized to dihydroxyacetone phosphate. Further studies have shown that dihydroxyacetone was oxidized by conversion to the phosphate, isomerization, and condensation to hexose diphosphate. The hexose diphosphate was dephosphorylated, and the hexose monophosphate formed was oxidized to 6-phosphogluconate which in turn was oxidized to pentose phosphate, the latter being converted to hexose phosphate through sedoheptulose phosphate and erythrose phosphate. There was no evidence for the oxidation of triose phosphate to the pyruvate level, since no acetate was formed although either lactate or pyruvate was oxidized to acetate. Thus hexose, pentoses, and trioses could be completely oxidized by this hexose monophosphate pathway without the participation of other systems. The glucose-6-phosphate dehydrogenase was found to be TPN linked; the 6-phosphogluconate dehydrogenase DPN linked.

Korn & Magasanik (64) have reported results which agree, in general, with the idea that two pathways exist. Glycerol oxidation by cells of *A. suboxydans* proceeded well in certain buffers at pH 6 or 8, but in the presence of acetate or dinitrophenol at pH 6 oxygen uptake ceased abruptly when the glycerol had been converted to dihydroxyacetone. Extracts contained a DPN-linked glycerol dehydrogenase which operated in the absence of phosphate at pH 6. The presence of other oxidizable substrates was required for the further oxidation of dihydroxyacetone at pH 6; at pH 8 the oxidation was insensitive to acetate or dinitrophenol and other oxidizable substrates were not required.

The finding by Hall, Kulka & Walker (65) that arabinose, ribulose, and tartaric acid were formed by heating Ca-2-ketogluconate indicates that the report of the formation of these compounds by *Acetobacter acetosum* from glucose in the presence of calcium carbonate was attributable to such decomposition.

The catabolism of various labeled glucoses by *P. fluorescens* was shown to be a complex affair, involving the Embden-Meyerhof path to a small extent, the hexosemonophosphate-pentose cycle for at least one half, and the Entner-Doudoroff scheme for one-third to one-half [Lewis *et al.* (66)] of the changes observed.

Glucose-grown *S. marcescens* were found by Wasserman, Hopkins & Seibles (67) to oxidize glucose, gluconate, and 2-ketogluconate without lag. Aldolase was absent and glucose was oxidized to 2-ketogluconate without evidence for phosphate intermediates. These reactions resemble those reported for *P. fluorescens* by Stokes & Campbell (68).

A series of papers from Wilson's laboratory demonstrated the complexity of the oxidation of carbohydrate by *Azotobacter vinelandii*. The failure to find phosphohexokinase indicated the absence of an Embden-Meyerhof route [Mortenson & Wilson (69)]. No 6-phosphogluconate dehydrogenase was demonstrated, and 6-phosphogluconate was shown to be split into glyceraldehyde-3-phosphate and pyruvate. The glyceraldehyde-3-phosphate could then be reconverted to hexose phosphate to provide more 6-phosphogluconate [Mortenson, Hamilton & Wilson (70)]. However ribose-5-phosphate was metabolized in the expected manner since sedoheptulose phosphate and triose phosphate accumulation could be demonstrated in cell-free extracts and ribose-5-phosphate and sedoheptulose phosphate are found in the cells. The hexose produced by further metabolism of sedoheptulose phosphate has not yet been identified, but the major area of uncertainty is how ribose-5-phosphate was formed since the usual conversion of 6-phosphogluconate to pentose phosphate was absent. The possible formation of pentose phosphate from triose phosphate and a 2-carbon fragment such as acetate was suggested [Mortenson & Wilson (71)].

Santer & Ajl (72) demonstrated that *P. pestis* ferments glucose-1-C¹⁴ in a manner compatible with an Embden-Meyerhof pathway and in a later

paper (73) have shown that, during growth especially, the appearance of $C^{14}O_2$ from glucose-1- C^{14} indicated the presence of the hexosemonophosphate-pentose scheme. This was confirmed by the demonstration of the enzymes.

The formation of D-ribose-5-phosphate from D-ribose and ATP in the presence of magnesium ions and extracts of *E. coli* has been reported by Heald & Long (74) and Long (75). Hochster (76) has demonstrated conversion of D-xylose to D-ribose phosphate and sedoheptulose phosphate by *P. hydrophila* extracts. *Pseudomonas saccharophila* oxidizes L-arabinose-1- C^{14} to yield one-half of the carbon and all the radioactivity in carbon dioxide; the remainder was assimilated [Weimberg & Doudoroff (77)]. In the presence of iodoacetate, the products were carbon dioxide and pyruvate. Arsenite induced the accumulation of α -ketoglutarate-1- C^{14} . The DPN-linked oxidation to L-arabonic acid followed by TPN- or DPN-linked oxidation to α -ketoglutarate seemed to be steps in the reaction chain. No phosphorylation could be demonstrated, nor any of the usual methods of α -ketoglutarate formation.

Evidence for the conversion of xylose to xylulose by *E. coli* and other observations on the oxidation of pentoses by cells grown on various media have been reported by Littauer, Volcani & Bergmann (78). Katznelson (79) has surveyed a collection of plant pathogenic and root nodule bacteria for their ability to convert 2-keto-3-deoxy-6-phosphogluconate to triose phosphate and pyruvate. Species of *Xanthomonas*, *Pseudomonas*, *Agrobacterium*, and *Rhizobium* have the ability, but *Corynebacterium* and *Erwinia* do not. *Xanthomonas* and *Pseudomonas* species also are able to oxidize 6-phosphogluconate. De Ley & Vandamme (80) tested a large collection of microorganisms grown with 2-keto-D-gluconate as a carbon source for the presence of a soluble kinase which would convert the keto acid to its phosphate ester. The kinase was found in species of *Pseudomonas*, *Xanthomonas*, *Escherichia*, *Aerobacter*, *Paracolobactrum*, *Serratia*, *Erwinia*, and *Bacillus*. The kinase was not found in species of *Agrobacterium*, *Corynebacterium*, *Schwannomyces*, *Debaryomyces*, *Lipomyces*, and *Candida*, nor in the groups Pyrenomycetes, Aspergillates, and Fungi Imperfecti although these organisms grew, and oxidized the keto acid after an induction period. The other organisms tested would not grow under the conditions tried.

The origin of deoxyribose in *E. coli* and T6R⁺ phage has been studied by Cohen & Lanning (81). The results indicated that the acetaldehyde-triose condensation could not be the only method of formation.

Cochrane (82) has shown that *Streptomyces coelicolor* contains many of the enzymes of the Embden-Meyerhof scheme, but neither cells nor extracts will ferment glucose or hexose diphosphate, probably because there is no mechanism present to regenerate DPN anaerobically. A hexosemonophosphate oxidation cycle has been shown in *Tilletia caries* by Newburgh Claridge & Cheldelin (83).

Carbon dioxide fixation by extracts of *Thiobacillus denitrificans* was increased by ATP ribose-5-phosphate; phosphoglycerate was formed [Trudinger (84)]. Santer & Vishniac (85) have shown that extracts of *Thiobacillus thioparus* fix labeled CO_2 in the presence of ribulose diphosphate, into the carboxyl groups of phosphoglycerate. Thus the carbon transformations of photo- and chemosynthesis appear to be similar.

Fermentations.—White, Steele & Pierce (86) have continued the investigation of the fermentation of galactose by *Streptococcus pyogenes* and found that, although the pH remained higher during galactose fermentation than during glucose fermentation, this pH difference does not account for the different fermentation pattern on galactose. Glycolysis by the Ford strain of *B. subtilis* (*B. licheniformis*) was stimulated by glutamate or glutamine, but inhibited by α -ketoglutarate. The products shifted from volatile acids to acetoin [Keynan, Strecker & Waelsch (87)]. Glycolysis by dried brewer's yeast was inhibited by sodium *N*-lauryl sarcosinate [Carbon and co-workers (88)]. Lichstein & Boyd (89) found that the rate of glucose oxidation by *Saccharomyces cerevisiae* was reduced by ultraviolet irradiation. Biotin, but not aspartate, stimulated oxidation by irradiated cells but only to the level of the normal. The rate of oxidation by normal cells increased following the addition of either biotin or aspartate, and the degree of stimulation increased with time.

Seigel & Smith (90) made use of the fact that dark and anaerobically adapted cells of *Rhodospseudomonas gelatinosa* metabolize the photosynthetic substrates to determine the nonphotosynthetic pathway of acetone metabolism. Substrates labeled with C^{14} were used, and the results indicated that acetone was carboxylated to acetoacetate, which was then decarboxylated to acetate. This mechanism was the same as that found photosynthetically and confirmed the assumption that light was not involved in the activation of the substrate.

Shankar & Bard (91, 92) have studied the magnesium and cobalt nutrition of *Clostridium perfringens*. Magnesium-deficient cells fermented glucose more slowly than did the normal cells. Several enzymes were found to be unchanged by the deficiency. Cobalt-grown cells produced only lactic acid from glucose. The effect was not a simple inhibition since it was not observed following the addition of cobalt to cell suspensions.

The effect of various ions on the glycolysis of a cell-free extract of *Lactobacillus arabinosus* has been reported by Clark & MacLeod (93). The extracts required ATP, Mn^{++} , and a monovalent ion. Surprisingly, since it inhibited in both growth and cell suspension experiments, NH_4^+ worked very well and, indeed, was irreplaceable under certain experimental conditions. Na^+ or Ca^{++} could inhibit competitively if insufficient NH_4^+ was present. Hexosemonophosphate or phosphorylated intermediates below it in the Embden-Meyerhof scheme spared the stimulatory effect and relieved the inhibition, which suggested that hexosemonophosphate formation was

the site of action. Further study of the apyrase of the cell-free extract showed it was stimulated by a variety of monovalent ions and inhibited by Mn^{++} , but the ion effect on glycolysis remains unexplained [Clark & MacLeod (94)].

Robertson & Boyer (95) have concluded that, since azide could not replace phosphate for glycolysis by yeast acetone powders, it did not liberate phosphate from phosphate esters; nor did it serve as a phosphate acceptor, since it did not stimulate the fermentation of hexosediphosphate that was limited by phosphate acceptors. Its action as a phosphorylation uncoupling agent must be sought elsewhere. Winder & Denny (96) reported that alumina-ground cells of *Mycobacterium smegmatis* used metaphosphate for the phosphorylation of glycerol in the presence of ATP.

Oxidations.—The endogenous respiration of *Chlorella vulgaris*, *Zygorhynchus moelleri*, and baker's yeast was examined by Moses & Syrett (97) using C^{14} -labeled cells. The amount of labeled CO_2 produced during the oxidation of unlabeled substrates indicated the amount of endogenous respiration that continued. In general, endogenous respiration was unaffected or somewhat stimulated but the authors warn against too facile interpretation of the data.

Goucher & Kocholaty (98) showed that repeated washing of *Azotobacter* cells with phosphate buffer inhibited the oxidation of succinate. Washing with a salt mixture or the addition of magnesium ions to the phosphate buffer prevents the inhibition. Cells washed in tris hydroxymethyl aminomethane were also deficient in oxidative ability, but no single ion could stimulate. Fe^{++} , Ca^{++} or Mo^{+6} plus Mg^{++} restored the activity and Mg^{++} plus $PO_4^{=}$ enhanced the activity. Mg^{++} was essential to several oxidations.

Labeled glucose was incorporated into streptomycin to the extent of 5 per cent if added after growth was maximal. The label was found in all parts of the molecule, but less was present in the guanidine moiety. Streptomycin was incorporated less efficiently than glucose, but N-methyl- C^{14} glucosamine was incorporated to the extent of 15 per cent [Hunter & Hockenhull (99)]. Ogur (100) has shown that the oxidation rate and the rate of aerobic carbon dioxide production by *Saccharomyces* on a cell-basis was proportional to the ploidy but was masked by using dry weight or cellular nitrogen as a basis.

Intracellular enzyme localization.—Wilson & Wilson (101) have purified the oxidative system of *A. vinelandii* fivefold. Succinate, malate, or lactate was oxidized by the preparation after exposure to 145,000 g for 3 hr. but, since no separation of activities by various treatments was obtained, activity associated with a particle was assumed. DPNH was oxidized and cytochrome-*c* could be reduced, but was only reoxidized if reduced by DNP systems, not if by other substrates or chemically. The *Azotobacter* cytochrome was spectrophotometrically similar to cytochrome-*c*, but differed enzymatically and physically. Repaske & Josten (102) have also described

the properties of the DPNH oxidase of *A. vinelandii* and of the associated cytochrome.

Tissières & Slater (103) have reported that particulate and supernatant fractions from *A. vinelandii* when combined yielded an increased oxygen uptake and phosphorylation of succinate. Dialyzed supernatant fluid was unable to fully stimulate phosphorylation but the effect on the oxidation rate was unchanged. Heated supernatant fluid had no stimulatory effect. The difficulties in locating enzymes within cells has been again illustrated by Linnane & Still (104). Cells of *S. marcescens* were disrupted by shaking with tiny glass beads in sucrose solutions. Succinic, α -ketoglutarate, lactic, and formic dehydrogenases were found to be particulate, but aconitase, fumarase, and isocitric and malic dehydrogenases might or might not be found associated with the particles depending on the shaking time and the sucrose concentration. Only the particles could react with oxygen, but attempts to reconstitute the separated systems were unsuccessful. Millman & Youmans (105) prepared from *Mycobacterium tuberculosis* var. *hominis* a red particulate fraction that contained cytochromes, most of the Krebs cycle enzymes, and a citrate-synthesizing system. This fraction did not require methylene blue for oxidation of many acids and could reduce tetrazolium. The supernatant fluid contained a variety of dehydrogenases whose relationship to the particulate enzyme is not clear since the treatment was drastic. Alexander & Wilson (106) have fractionated *A. vinelandii* extracts and have shown that the systems that connect to oxygen are particulate. The tricarboxylic acid cycle enzymes were not all particulate since isocitric dehydrogenase, aconitase, and α -ketoglutarate oxidase were found in the soluble fraction. The enzymatic activity of the particulate fraction of *E. coli* on many substrates has been described by Asnis, Vely & Glick (107). DPNH was oxidized by the particles, but TPNH was not unless menadione and a flavoprotein fraction was added.

Hydrogenase, hydrogenlyase, and one-carbon compounds.—Korkes (108) has found that extracts of *Clostridium kluveri* reduced pyridine nucleotides in the presence of H_2 . By means of the appropriate enzymes various substrates could be reduced. A heat stable, alkali labile cofactor was required by dialyzed extracts.

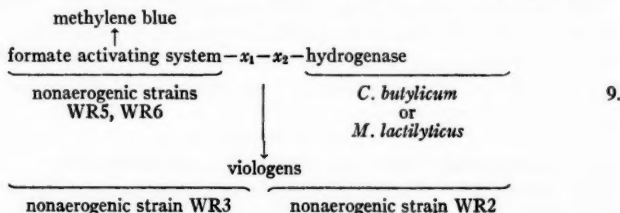
Peck & Gest (109) have recommended the use of hydrogen production from reduced methyl viologen as an assay for hydrogenase. The complexity of the system was indicated by the observations that *Clostridium butylicum*, which did not reduce methylene blue in the presence of hydrogen, evolved hydrogen vigorously from reduced methyl viologen; several other organisms could reduce methylene blue but could not evolve hydrogen from reduced methyl viologen. *Micrococcus lactilyticus* had both activities but extracts could be fractionated to preparations that only reduced methylene blue.

The inhibition of the soluble hydrogenase of *Desulphovibrio desulphuricans* by methylene blue and, to a much lesser extent, by benzyl viologen or

methyl violet was reported by King & Winfield (110). The particulate hydrogenase, presumably screened by the structure, was not so inhibited. The authors recommended the exchange reaction or hydrogen evolution from reduced benzyl viologen as the most satisfactory assay for hydrogenase. Whiteley & Ordal (111, 112) have shown that the soluble hydrogenase of *M. lactilyticus* would reduce methyl viologen or xanthine in the presence of H_2 . The reduction of methylene blue required the addition of heat labile materials such as *M. lactilyticus* particles, *E. coli* particles, or dilute *E. coli* extracts which had only low hydrogenase activity alone. Methylene blue inhibited the reduction of benzyl viologen by the soluble hydrogenase. Marked synergism between the soluble and particulate preparations from *E. coli* for not only hydrogenase, but also for formic dehydrogenase, hydrogenlyase, and various oxidations has been reported by Swim & Gest (113).

Gest & Peck (114) have produced further evidence that formic hydrogenlyase is not a single enzyme system from a study of cell suspensions and cell-free extracts of normal and nonaerogenic strains of *E. coli*. The combination of extracts of nonaerogenic strains which showed only formic dehydrogenase activity with that of one which showed only hydrogenase activity yielded considerable hydrogenlyase activity. The further complexity of the system was indicated by the finding that some of the extracts of nonaerogenic strains reduced methylene blue, but not the low potential, one-electron viologens, in the presence of formate. Such extracts could not be used to reconstitute hydrogenlyase, so an essential carrier x_1 has been postulated. A second essential carrier x_2 was postulated because extracts of *C. butylicum* or *M. lactilyticus* which actively produced hydrogen from pyruvate could not be used to provide the hydrogenase to reconstitute hydrogenlyase. The postulated carrier x_2 could be replaced by methyl viologen and apparently was not required for hydrogen production from pyruvate.

The hydrogenlyase system can be represented by the following scheme:



The characteristics of the hydrogenase and also observations on carbon dioxide fixation by *Hydrogenomonas ruhlandii* nov. spec. have been reported by Packer & Vishniac (115, 116). Exchange of carbon dioxide with pyruvate in the presence of suspensions of *Clostridium acidurici* or several other microorganisms has been reported by Sagers & Beck (117). The cofactor requirements for the more rapid exchange of carbon dioxide with pyruvate

in the presence of cell-free extract of *C. butyricum* were found to be DPT, CoA, and phosphate or arsenate by Wolfe & O'Kane (118). The exchange reaction system was more stable than the pyruvate decomposition one and was inhibited by electron acceptors such as furacin. A slow and labile exchange reaction occurred with acetate and this could be stimulated by phosphate and CoA, but no exchange with acetyl phosphate, and presumably acetyl coenzyme A, since phosphotransacetylase and CoA are present, could be demonstrated. Novelli (119, 120) used extracts of alumina-ground *C. butyricum* or *M. lactilyticus*, in contrast to the other types of cell-free extracts used by Wilson, Krampitz & Werkman, (121) and Wolfe & O'Kane (118), and was able to demonstrate formate exchange with pyruvate by each organism. The new scheme suggested, involving the formation of a two-carbon compound of the acetate level of oxidation and a one-carbon compound which can be converted to formate or hydrogen and carbon dioxide, may explain the complex cofactor requirement for the exchange reaction mentioned above. It explains the dependency of rapid one-carbon exchange reactions on reducing systems, the inhibition by electron acceptors, and the effect of pH on the reaction.

Stoppani, Fuller & Calvin (122) have compared carbon dioxide fixation in the light and the dark by *Rhodospseudomonas capsulatus*. Light was much more efficient than hydrogen oxidation in the dark for carbon dioxide fixation; in the light the bulk of the CO₂ was fixed through phosphoglycerate whereas in the dark fixation through Krebs' cycle intermediates predominated.

Miscellany.—The aerobic inhibition of glucose utilization by yeast caused by Decarborane in the presence of azide has led Hill, Hawkins & Zak (123) to postulate that the site of action was on fermentation. Penicillin added to *Micrococcus pyogenes* var. *aureus* inhibited carbon dioxide production from glutamine, and stimulated carbon dioxide production from glucose, although the total carbon dioxide production was not changed (Bidwell *et al.* (124). The effect of phosphate concentration (as distinguished from pH changes) on the production of various organic acids from molasses by *A. niger* was reported by Martin & Steel (125). Oxalic acid only accumulated if phosphate was absent, but gluconic acid and 5-ketogluconic acid accumulation depended on phosphate. Biotin deficiency caused the accumulation of dimethylpyruvic acid, as well as pyruvic acid and α -ketoglutaric acid, in growing cultures of *Piricularia oryzae*. Deficiency of other B vitamins caused keto acids to accumulate but not dimethylpyruvic acid [Katsuki (126)].

Cell-free extracts of *Microsporon canis* oxidized several substrates, but only decarboxylated oxalacetate if DPT and DPN were added [Chattaway, Thompson & Barlow (127)]. Zakrzewski & Nichol (128) have demonstrated the formation of radioactive citrovorum factor by the incubation of pteroylglutamic acid with labeled formate and *S. faecalis* cells.

The conversion of glucose to acetate and lactate, succinate, and other nonvolatile acids during the growth of *Strigomonas oncopelti* has been reported by Clausen (129). Ryley (130, 131) has reported much detailed information about the metabolism of *S. oncopelti* and *Trichomonas foetus*.

The conversion of sedoheptulose-1,7-diphosphate to 5-dehydroshikimic acid by extracts of *E. coli* has been shown to proceed through the conversion of the sedoheptulose diphosphate to D-erythrose-4-phosphate and phosphoenolpyruvate, which condenses to form 5-dehydroshikimic acid. The intermediate formation of 2-keto-3-deoxy-7-phospho-D-glucoheptonic acid, analogous to the formation of oxalacetate from phosphoenolpyruvate and carbon dioxide, was suggested and would explain the configurational difference between sedoheptulose-1,7-diphosphate and 5-dehydroshikimic acid [Srinivasan, Katagiri & Sprinson (132)]. The suggestion that shikimic acid is a precursor of lignin in higher plants indicates further biological importance for this compound [Brown & Neish (133)].

An isotopic study of the conversion of anthranilic acid to indol by *E. coli* further clarifies the relationship of carbohydrate metabolism to synthetic reactions. The two carbons added to anthranilic acid were found to be labeled in a fashion that indicated the participation of both the Embden-Meyerhof and the hexosemonophosphate-pentose pathways [Yanofsky (134, 135)].

CARBOXYLIC ACIDS

The chief activity in this area has been further studies with various microorganisms to demonstrate the reactions of the tricarboxylic acid cycle. Acetate oxidation continued to receive much attention since other mechanisms, particularly the dicarboxylic acid cycle, have been suggested for its oxidation. As the reports below will indicate, the position is not yet clear. The elegant isotopic experiments of Krampitz and his colleagues, and the elimination of one of the principle proponents of the dicarboxylic acid cycle, considered with the results of work previous to 1955, would seem to establish the tricarboxylic acid cycle. But the discovery by Seaman (136, 137) of the enzyme which catalyses the postulated first reaction of the dicarboxylic cycle indicates that further work will be required. Additional work is also required to decide the ratio of energy production to intermediate production in various organisms.

Saz & Krampitz (138), continuing their studies of the tricarboxylic acid cycle, showed that, although whole cells of *M. lysodeikticus* were unable to oxidize citrate, cell-free extracts rapidly oxidized it to α -ketoglutarate. Acetate-2- C^{14} was converted to α -ketoglutarate to the extent of 91 per cent in a system containing fumarate and arsenite in which negligible recycling occurs. To further emphasize the difference between whole cells and cell-free extracts, it was shown that succinate oxidation was insensitive to malonate in whole cells, but was inhibited in cell-free extracts. De Moss, Swim & Krampitz (139) have studied the oxidation of labeled acetate by baker's yeast and found the α -ketoglutarate, citrate, fumarate, succinate, and malate highly

labeled and in isotopic equilibrium with each other. The distribution of the isotope in citrate and succinate was that predicted from the tricarboxylic acid cycle and the authors concluded that the cycle was not only operative but was a considerable energy source. An excretion of α -ketoglutarate by suspensions of a vibrio that had been grown on acetate was reported by Dagley & Patel (140). Other growth substrates yielded cells capable of oxidizing acetate but no α -ketoglutarate was excreted. Other fatty acids gave results similar to those obtained with acetate, presumably being first converted to acetyl-coenzyme A. The authors conclude that acetate was metabolized to a C_4 compound which was converted to α -ketoglutarate.

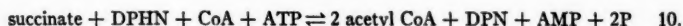
Beck & Lindstrom (141) have demonstrated that cells and cell-free extracts of *Bacillus cereus* show the oxidative reactions of the tricarboxylic acid cycle. Cell-free extracts were unable to oxidize acetate so apparently the necessary activation system was lost. The adaptive nature of some of the tricarboxylic acid cycle enzymes in *P. pestis* was described by Englesberg & Levy (142). Anaerobically-grown cells were unable to oxidize acetate and oxidized glucose slowly and incompletely with the accumulation of various products. Aerobically-grown cells, however, oxidized pyruvate, acetate or C_4 -dicarboxylic acids rapidly, and glucose was oxidized rapidly without accumulation of fermentation products. Aeration of anaerobically-grown cells in a nutrient medium, but under conditions that precluded mutation and selection as a mechanism, produced a conversion to the pattern shown by aerobically grown cells. This adaptation involved the appearance of increased amounts of phosphotransacetylase, condensing enzyme, isocitrate dehydrogenase, aconitase, fumarase, malic dehydrogenase, cytochrome, and the enzyme systems that oxidatively decarboxylate pyruvate and α -ketoglutarate. This was not a general, nonspecific increase in synthesis since activity of several other enzymes remained the same or decreased. An inhibitor phenomenon was not involved since anaerobic extracts did not inhibit the increased activity of aerobic extracts. The changes implicated the tricarboxylic acid cycle, but the aerobic extracts would not oxidize acetate even though the enzymes necessary to activate acetate were present (acetokinase and phosphotransacetylase). The tricarboxylic acid cycle acids are oxidized incompletely with the accumulation of acetate. The authors attributed these results to the rapid destruction of oxalacetate and the large concentrations of acetate required to produce a reasonable rate of activation.

Aubel & Prieur (143) confirmed that *E. coli* may be adapted to acetate and citrate oxidation either by growing it aerobically or by aerating anaerobically-grown cells. The adaptive patterns of the genus *Azotobacter* have been studied by Schutter & Wilson (144) and were recommended as a means of differentiating the *Azotobacter agile* and the *A. vinelandii* groups. The authors made a general case for the modernization of the physiological tests used in identifications.

In a study of the action of chloramphenicol on *P. fluorescens*, Kushner

(145) showed that the oxidation of α -ketoglutarate, succinate, fumarate, and malate was inhibited in cells and cell-free extracts, but citrate oxidation, among others, was not. Many individual steps of the tricarboxylic acid cycle were not inhibited so it was suggested that the cycle may not be the pathway normally used to oxidize those dicarboxylic acids whose oxidation was inhibited by chloramphenicol. Ajl (146), who supported the idea that a dicarboxylic acid cycle operated in *E. coli* rather than a tricarboxylic acid cycle, has reconsidered and found in experiments using large numbers of cells that citrate and succinate equilibrated with respiratory CO_2 and that dried cells incorporated acetate into the tricarboxylic acid cycle intermediates. Earlier experiments, using smaller numbers of cells and added carriers gave erroneous results because the added carriers did not equilibrate with the metabolically active compounds. No C_2 to C_4 condensation was demonstrable and the presence of the dicarboxylic acid cycle is discounted.

A common objection to the dicarboxylic acid cycle has been the failure to demonstrate the first reaction: the condensation of two molecules of acetate to form succinate. However, Seaman (136) and Seaman & Nashke (137) described an enzyme from *Tetrahymena* which carried out the following reaction:



The enzyme also occurs in *E. coli* and this enzyme must be kept in mind before the dicarboxylic acid cycle is completely discarded. The enzyme is interesting in its own right since it provides a method of regenerating DPN from DPNH produced by glycolytic reactions.

Gilmour *et al.* (147) found all the tricarboxylic acid cycle reactions in cells of *Streptomyces griseus*. The concentration of succinate, α -ketoglutarate, and citrate had to be increased in order to adequately demonstrate their oxidation, suggesting that permeability differences operated in this organism, too. Glutamic acid labeled in the carboxyl groups in the proportions required by the operation of the tricarboxylic acid cycle accumulated during acetate- 1-C^{14} oxidation. Another paper by Butterworth, Gilmour & Wang (148) showed that C^{14}O_2 fixation by growing cells could be accounted for by the operation of $\text{C}_2 + \text{C}_1$ condensations and the tricarboxylic acid cycle. *Streptomyces nitificans*, which grows on urethan as a nitrogen and carbon source has been shown to oxidize the tricarboxylic acid cycle intermediates by Schatz, Mohan & Trelawny (149).

Many of the reactions of the tricarboxylic acid cycle were shown to occur in cell suspensions of *Zygorrhynchus moelleri* and preparations from cells frozen in liquid nitrogen. Once again succinate oxidation was sensitive to malonate only in cell preparations, and a low pH was required before cells oxidized many of the acids. Since only 5 per cent of the oxidative capacity was present in the frozen preparations the author, Moses (150), wisely avoided any conclusions about the quantitative significance of the reactions demonstrated.

An acetate-requiring mutant of *Neurospora crassa*, which accumulated pyruvate and acetoin when cell suspensions were shaken with buffer and acetate or glucose, has been shown by Strauss (151) to incorporate the methyl carbon of acetate into pyruvate. Carbon dioxide was also incorporated into pyruvate. When fluoroacetate inhibited acetate oxidation and growth, citrate accumulated. These data were interpreted in favor of a tricarboxylic acid cycle, but a dicarboxylic acid cycle was not excluded.

Cabelli (152) has studied the adaptation of *Klebsiella pneumoniae* to various substrates during growth, and the oxidation of these compounds by cells adapted by growth on the various compounds. Citrate, although it supported growth after a lag, was not oxidized even by citrate-grown cells. From this and similar data, it was concluded that a tricarboxylic acid cycle was not present. This conclusion may be premature, since the failure of adaptation experiments to indicate the presence of enzymes within cells has been well documented, especially by Barrett & Kallio (153).

Ramakrishnan (154) has shown that cell-free extracts of *A. niger* could carry out the reactions of the tricarboxylic acid cycle, were able to activate acetate, and contained phosphotransacetylase. Further study (155) has shown that as citric acid accumulated, the level of condensing enzyme increased but that of aconitase and of isocitric dehydrogenase decreased. The accumulated citric acid inhibited isocitric dehydrogenase.

Itaconic acid accumulation by *Aspergillus terreus* has also been studied by Larsen & Eimhjellen (156) with particular attention to the requirement for a low pH. Mycelia grown at 2.1 would convert glucose to itaconic acid at either pH 2.1 or 6.0, so the failure to accumulate itaconic acid at pH 6.0 was not due to preferential utilization at that pH.

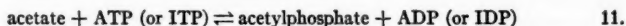
The acceleration of citrate oxidation in yeast cells by aspartic acid was reported by Foulkes (157). DPN or TPN was also required but the effect seemed not to be related to cycling of the coenzyme or transamination.

The effect of freezing yeast cells with solid CO₂ has been investigated by Hansen & Nossal (158). The interest in these preparations was related to their use by Krebs, Gurin & Eggleston (159) in experiments that led to the conclusion that the tricarboxylic acid cycle functioned in yeast to furnish intermediates, not as a major path of oxidation. The frozen cells became permeable to added organic acids and lost many of their internal constituents. Linnane & Still (160) were able to prepare mitochondria from yeast. Acetate was oxidized if catalytic amounts of citrate or α -ketoglutarate were added, but was not oxidized in the presence of succinate, malate, or malate plus ATP. Pyruvate oxidation, however, was sparked by any of the above substances. Apparently acetate is activated in this case by exchange with succinyl-coenzyme A produced from α -ketoglutarate or citrate. Wiame & Burgeois (161) suggested that the biosynthetic function of the tricarboxylic acid cycle was the basis for the CO₂ requirement of many microorganisms. That is, the withdrawal of compounds from the cycle is compensated by CO₂ fixation to keep the cycle going. The isocitratase of *E. coli* that catalyzes the

formation of succinate and glyoxalate from *iso*-citrate was discussed by Wong & Aji (162).

The failure of a *Chlamydomonas* mutant to assimilate acetate although acetate was oxidized has been reported by Lewin (163). Samson, Katz & Harris (164) show that short chain fatty acids inhibit respiration, fermentation, and phosphate accumulation in yeast cells and cell-free extracts.

The properties of the acetate-activating enzyme of the type found in many bacteria were reported by Rose *et al.* (165). This kinase catalyzed the reaction:

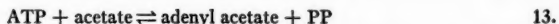


and required magnesium or manganese ions. *Rhodospirillum rubrum*, however, activated acetate in the manner that yeast, higher plants, and animals do [Eisenberg (166)], that is by the reaction:



This reaction was also demonstrated in *P. fluorescens* by Hayaishi (167).

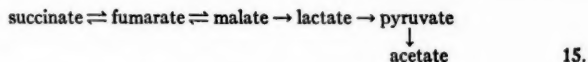
The mechanism of this acetate-activating reaction was thought to involve formation of an enzyme-adenosine monophosphate and pyrophosphate, followed by the exchange of CoA for adenosine monophosphate and the subsequent formation of acetyl CoA and enzyme. The experimental evidence for this mechanism was the rapid exchange of the pyrophosphate group of ATP with inorganic pyrophosphate in the presence of an enzyme from yeast [Jones *et al.* (168)]. The discovery by Berg (169) that acetate acted catalytically in the pyrophosphate exchange has suggested the following mechanism:



Synthetic adenylyl acetate yields acetyl CoA when incubated with the yeast enzyme and CoA.

Brady & Stadtman (170) have described the thioltransacetylases A and B of *C. kluyveri* extracts. These enzymes transferred the acetyl groups of acetyl CoA to other thiol compounds.

Growing cultures of *Desulphovibrio desulphuricans* reduced sulfate to hydrogen sulfide and converted malate, fumarate, succinate, lactate, or pyruvate to acetate. Suspensions of cells grown on the various substrates were studied by Grossman & Postgate (171) and the following sequence was suggested:



The first step in the sequence may be the link to sulfate reduction.

Wolin, Evans & Niven (172) have shown that the inhibition of pyruvate oxidation by cell suspensions of *Micrococcus pyogenes* var. *aureus* by methylene blue and oxygen could be reversed by thiamine or thiamine pyrophosphate, plus magnesium ions. When this pyruvate oxidation system was inhibited, pyruvate was removed by acetoin formation. At high substrate levels, both systems functioned.

The activation of cell-free extracts of cells of *S. faecalis*, grown in the absence of lipoic acid, by lipoic acid has been studied by Leach, Yasunobu & Reed (173) using the α -ketobutyrate oxidation system. Activity of the oxidative system paralleled the uptake of lipoic acid-S³⁵; magnesium ions, phosphate, and two protein components were required for binding. Dolin (174) described the oxidation of diacetyl to acetate in the presence of ferricyanide, DPT, magnesium ions, and lipoic acid. In the absence of ferricyanide, acetoin and diacetyl methyl carbinol were formed.

The complex nature of the decarboxylation of malonate to acetate and CO₂ by *P. fluorescens* was shown by the observations of Wolfe, Ivler & Rittenberg (175) to involve an oxygen uptake per mole of malonate greater than per mole of acetate. Thus a simple decarboxylation to acetate and CO₂ would not explain the reaction. The establishment of the intermediate role of acetyl CoA in the reaction by Hayaishi (167) seemed to offer an explanation, but cell-free extracts which oxidize malonate after decarboxylation has occurred (and which could be demonstrated to produce acetyl CoA), would not oxidize acetate. Clearly some compound other than acetyl CoA was being oxidized and Wolfe *et al.* (175) suggested a scheme involving diactivated malonate. Since the oxygen consumed was not stoichiometrically related to the malonate added it was suggested that the diactivated compound was oxidized. Phares & Carson (176) have prepared an enzyme from *Propionibacterium* that converted succinyl CoA to propionyl CoA and a 1-carbon compound that was not carbon dioxide, but was converted to carbon dioxide by a preparation from *Veillonella gazogenes*. The possible relationship of this observation to the malonate system, and the conflicting data about the malonate system have been discussed by Delwiche (177).

Jayasuriya (178) has described an organism, probably a pseudomonad, that grew on and was able to oxidize oxalate. Other acids such as glycollate, lactate, pyruvate, malate, or succinate supported growth. *Mycobacterium lacticola*, isolated from the intestine of earthworms by Khambata & Bhat (179), also decomposed oxalate. The oxidation of propionate by a marine bacterium has been described by Tomlinson & MacLeod (180). The oxidation of caprate by cell-free extracts of *P. fluorescens* was considered by Ivler, Wolfe & Rittenberg (181) to be in accord with the well-known scheme involving CoA and ATP for activation and β -oxidation. Webley, Duff & Farmer (182) showed that cells of *Nocardia opaca* converted phenyl-substituted fatty acids with an odd number of carbon atoms in the fatty acid chain to benzoic acid through cinnamic acid as an intermediate. Fatty acid

side chains with an even-number of carbon atoms were apparently converted to phenyl acetic acid which was metabolized further. The accumulation of *o*-hydroxyphenyl acetic acid was attributed to a side reaction. An actinomycete capable of using cyanide as a sole source of carbon and nitrogen has been described by Ware & Painter (183).

HIGHER SACCHARIDES

Transglycosidases.—The reversibility of action of the levansucrase of *B. subtilis* has been demonstrated by Péaud-Lenöel & Dedonder (184). This confirms the previous demonstration of reversibility by Doudoroff & O'Neal (185) who used invertase to remove the sucrose formed. Péaud-Lenöel (186) also synthesized other disaccharides from levan and aldoses in the presence of levansucrase:

levan + glucose \rightarrow α -D-glucosido-1,2- β -D-fructofuranoside	16.
levan + galactose \rightarrow α -D-galactosido-1,2- β -D-fructofuranoside	17.
levan + arabinose \rightarrow α -D-arabinosido-1,2- β -D-fructofuranoside	18.
levan + xylose \rightarrow α -D-xylosido-1,2- β -D-fructofuranoside	19.

A study of the dextran-synthesizing system of *L. mesenteroides* by Tsuchiya *et al.* (187, 188) showed that the yield and molecular weight of the product was affected by the initial sucrose concentration. The addition of maltose produced low molecular weight polymer and oligosaccharides. Low molecular weight polymer added to an active system was increased in molecular weight. The authors proposed a mechanism analogous to the familiar one of chain initiation, propagation, and termination that was more useful in explaining observations than previous proposals:

Initiation:	
enzyme + acceptor \rightarrow (enzyme-acceptor)	20.
Propagation:	
(enzyme-acceptor) + sucrose \rightarrow (enzyme-acceptor-glucose) + fructose	21.
Termination:	
(enzyme-acceptor-glucose) \rightarrow enzyme + acceptor-glucose	22.

Bailey *et al.* (189), and Barker *et al.* (190) studied a similar system in *Betacoccus arabinosus* (synonym = *Leuconostoc mesenteroides*?) and found an α -1:6-dextran, with branches at carbons one and three, whose average chain length could be increased from 6-7 to 40-50 glucose units by a magnesium ion deficiency during synthesis. The formation of oligosaccharides and polymers of lower molecular weight in response to the addition of several sugars in varying concentrations was reported. The results seem to be in accord with the scheme above. Several organisms isolated during a program designed to select from soil producers of polysaccharides of certain molecular weight ranges were studied by Mattoon and coauthors (191). The strain chiefly used was identified as *B. subtilis*. Levan was produced from sucrose and was ac-

accompanied by the hydrolysis of sucrose and levan. The addition of primers increased the yield and gave a more uniform molecular weight. Many characteristics of the system and the polysaccharide produced were reported. In general the levan-synthesizing system resembled the dextran-synthesizing system.

Oligosaccharide formation by transglycosidation with enzymes from *Aspergillus oryzae* has been reported by Pazur (192) and from *Penicillium chrysogenum* by Saroja, Venkataraman & Giri (193). The formation of 4-O- β -D-galactopyranosyl-N-acetyl-D-glucosamine from lactose and N-acetyl-D-glucosamine by intact cells of *Lactobacillus bifidus* was accomplished by Zilliken, *et al.* (194). The synthesis of 6- β -D-galactopyranosyl-N-acetyl-D-glucosamine from N-acetyl-D-glucosamine and β -phenyl-D-galactoside by a cell-free preparation from *E. coli* was reported by Kuhn, Baer & Gauhe (195).

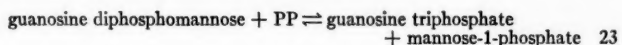
Hydrolases.—Myrbäck & Willstaedt (196) reported the inhibition of yeast invertase by the uranyl ion, which appeared to act by combining with the carboxyl groups of the proteins since the degree of inhibition increased with increase of pH. These authors (197) localized invertase on the outside of the cell membrane since it was destroyed by an acid treatment that did not prevent fermentation, and since the insoluble invertase was attached to fragments of the cell membrane when the cell was disrupted. The transglycosidase activity of invertase was noted. The localization of invertase agreed with previous work and with the treatment of Best (198, 199), who concluded from an examination of the effect of temperature and substrate concentration on reaction rates that invertase was on the outside of the cell membrane and hexokinase was inside.

A systematic study of the hydrolysis of cellulose by an enzyme derived from *Myrothecium verucaria* was carried out by Whitaker (200). The chain-splitting appears random and the turnover number increases with the size of the oligosaccharide used as substrate. Hammerstrom, Claus, Coghlan & McBee (201) showed that both *Clostridium thermocellum* and *Cellulomonas flavigena* produced cellulase when grown on carbohydrates other than cellulose or its hydrolysis products. Considerably more cellulase was produced during growth on cellulose or its hydrolytic products, however. Several microorganisms are able to grow on cellulose or cellobiose, but not glucose. The demonstration of a cellobiose phosphorylase produced by *C. thermocellum* by Sih & McBee (202), offered a partial explanation of the observations on growing cultures. The hydrolysis of xylan by *A. foetidus* and the effect of various treatments on the products produced was reported by Whistler & Masak (203).

The α -amylases produced at 35°C. and 55°C. by the facultatively thermophilic bacteria, *Bacillus coagulans* and *Bacillus stearothermophilus* were studied by Campbell (204). Their properties are discussed, the chief difference being the greater heat resistance of the enzyme produced at the higher temperature. Yaphe & Baxter (205) have studied an extracellular enzyme

produced by a marine bacterium. The enzyme hydrolyzed carrageenin, a polysaccharide of red algae, but not agar. Microorganisms centrifuged from rumen liquor were shown by Marsh (206) to contain β -glucuronidase.

Monosaccharide transformations.—Hansen & Freedland (207, 208) found that crude extracts of *Lactobacillus bulgaricus* or *Streptococcus fragilis* interconvert galactose-1-phosphate and glucose-1-phosphate in the presence of either uridine diphosphohexose or uridine triphosphate. A fraction from the *L. bulgaricus* extract responded only to uridine diphosphohexose, whereas a fraction from *S. fragilis* could not interconvert the sugar phosphate plus uridine triphosphate to uridinediphosphohexose. Munch-Petersen (209) has demonstrated the reaction:



The enzyme was found in *S. fragilis* and might function as a mannose donor in polysaccharide synthesis.

Polysaccharide synthesis.—Considerable work on the polysaccharides of microorganisms was reported. Wilkinson, Dudman & Aspinall (210) found the extracellular polysaccharide of *A. aerogenes* to be 50 per cent D-glucose, 10 per cent L-fucose, and 29 per cent unidentified uronic acid. The composition was independent of the growth carbohydrate. The qualitative composition of the carbohydrate of the "O" somatic antigens of several gram-negative rods was reported by Davies (211). Davies, Morgan & Record (212) studied the degraded haptenic carbohydrate of the "O" somatic antigen of *Shigella dysenteriae* in some detail and found 27.5 per cent acetylglucosamine, 33 per cent rhamnose, and 27 per cent galactose with a molecular weight of 25×10^3 . Eagon & Dedonder (213) found the capsular polysaccharide of *P. fluorescens* to contain 71 per cent glucose, 24 per cent glucosamine, and 5 per cent fucose. A study of several strains of *P. fluorescens* by the same authors (214) revealed great qualitative variation in the composition of the capsular polysaccharides. The capsular polysaccharide of *P. aeruginosa* was shown by Warren & Gray (215) to be easily washed from the cell surface and to be depolymerized by hyaluronidase. De Leizaola & Dedonder (216) found sugars in the proportion of 12 glucose, 3 galactose, 2 rhamnose, and 1 mannose in the polysaccharide of strain Soya of *Rhizobium*. The composition of the polysaccharides of other strains was complex and variable, but uronic acids were never found. The authors suggest that presence of uronic acids in the polysaccharides studied by Schlüchterer & Stacey (217) was attributable to the more complex medium used for growth.

Continuing a study of hyaluronic acid synthesis, Dorfman, *et al.* (218) reported that, although the appearance of the label from carboxyl-labeled N-acetylglucosamine in hyaluronic acid was inconclusive because the substrate was deacetylated and the acetate used in synthesis; C¹⁴-N¹⁵-glucosamine is incorporated directly by group A streptococci. Glucosone was elim-

inated as a possible glucosamine precursor by experiments with labeled glucosone. Dorfman *et al.* (219) reported that, although labeling occurred, it was a result of the degradation of glucosone to simpler compounds which were resynthesized. The formation of glucosamine from ammonia and glutamate or glutamine by group A or C streptococci and the inhibition of the ammonia and glutamate reaction by methionine sulfoxide was demonstrated by Lowther & Rogers (220). Cell-free extracts capable of forming glucosamine from glutamine were obtained. Glucosamine degradation by *S. marcescens* was found by Payne *et al.* (221) to be adaptive and to proceed through a deamination and direct amination of α -ketoglutarate by the ammonia produced.

The conditions that led to the formation of an intracellular iodophilic polysaccharide by group D rumen streptococci have been studied by Hobson & Mann (222). The properties of the amylase of certain strains of *S. pyogenes*, and the synthesis of an amylose under proper conditions by a system similar to the amylose system of *E. coli* was reported by Crowley (223) and Crowley & Jevons (224).

Barker *et al.* (225) showed that a streptococcus isolated from home-made elderberry wine produced from sucrose an α -linked 1:6 dextran with a small amount of branching. The value of infrared spectra to indicate linkages and components of polysaccharides was stressed. Butler & Stacey (226) reported methylation studies of Type II pneumococcus polysaccharide which indicated a highly-branched rhamnose, glucose, and glucuronic acid structure. Hoeprich (227) has studied the conditions for the production of C^{14} -labelled Type II pneumococcus polysaccharide. Many carbon compounds served as a source of label, but acetate was best.

The synthesis of C^{14} -labeled cellulose by *Acetobacter xylinum* from glucose-2- C^{14} confirmed the previous experiments with glucose-1- C^{14} . Minor, Greathouse & Shirk (228) demonstrated that the specific activity of the cellulose produced was lower than that of the substrate, and considerable labeling appeared in other than the 2-position, thus showing that a direct conversion was not involved.

The structure of yeast glycogen was studied by two groups. Peat, Whelan & Edwards (229) showed by partial hydrolysis and isolation of the oligosaccharides from charcoal-celite columns that baker's yeast glycogen contained only α -1:4 and α -1:6 glucosidic linkages. They preferred this method to classical methylation or periodate oxidation. Manners & Maung (230) found brewer's yeast glycogen to have a molecular weight of about 2 million, an average chain length of 13 glucose residues, a β -amylolysis limit of 44 per cent, and a phosphorolysis limit of 23 per cent. The linkage was 1:4 and 1:6. Hence the product is a typical glycogen, although slightly different from that of baker's yeast.

Barker *et al.* (231) thought streptomycin might affect enzymes responsible for synthesis and degradation of polysaccharides owing to the structural

relationship or the ability to serve as donor or receptor in transglycosylations. Although inhibitions were observed, they were slight and only occurred at high concentrations.

The red marine alga *Tridophycus flaccidum* showed the same general picture of CO₂ assimilation as *Chlorella* and higher plants, but Bean & Hassid (232) showed that α -D-galactosyl-2-glycerol which is analogous to sucrose in higher plants, appeared to be the main carbohydrate reserve. The data suggested that uridine diphosphogalactose was involved in the synthesis. The glycogens of two trichomonads, *Trichomonas foetus* and *T. gallinae*, were shown by Manners & Ryley (233) to be typical animal glycogens with a molecular weight of about 3 million per unit chain of 9 to 15 units, and a 51 to 60 per cent β -amylolysis limit.

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PROTOZOA AND ALGAE¹

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This review deals with various studies in recent years on sex, reproduction and cytogenetics of Protozoa and Algae as far as they were not discussed in the summaries prepared by Wenrich (1947), Sonneborn (1949) and Fauré-Fremiet (1953) in the *Annual Review of Microbiology*. Generally, publications which appeared before 1951 will not be considered. Terminology is the same as used by the reviewer in his book (60).

PHYTOMONADINA

The questionable results of Moewus, which in many cases could not be reproduced by others, have stimulated numerous recent investigations on sexuality and genetics of *Chlamydomonas*. The papers so far published are confined to isogamous dioecious (heterothallic) species in which sex-determination is genotypic. In meiosis, which is zygotic and consists of two divisional steps, zoospores (gones) are formed, half of which belong to one sex and half to the other.²

The sexual phenomena in *Chlamydomonas* are induced by making vegetative cells sexually active, i.e. by transforming them directly into gametes. The first requirement for this is a liquid medium in which the cells form flagella and can move freely. Sager & Granick (81) demonstrated that in *Chlamydomonas reinhardtii* sexual activation always takes place when the cells have no nitrogen compounds available for growth. Supplied again with nitrogen compounds, they change back into vegetative cells. Although light is not a prerequisite for the activation of the cells, it favors copulation. Sager & Granick suggest that the role of light in increasing copulation is caused by nitrogen depletion through photosynthesis or the reactions following it. Bernstein & Jahn (3) proposed the same explanation for *Chlamydomonas eugametos* and *Chlamydomonas moewusii*. This idea agrees with Lewin's demonstration in *C. moewusii* (71) that the action spectrum of light for mating corresponds approximately to the absorption spectrum of chlorophyll.

These authors assume that light affects both sexes in the same way, but Förster & Wiese (33) found that in *C. eugametos* the influence of light on mating is sex-specific. Although both kinds of gametes are motile and able to

¹ The survey of literature pertaining to this review was completed in November, 1955.

² In American literature it has become common to speak of "mating types" instead of different "sexes," even when there is a bipolar difference between true sex cells (gametes). This terminology only leads to confusion, since in the same breath one speaks also of sex-differentiation, sex-reaction, etc. In addition, it suggests a similarity with the mating types of ciliates which, as is known, are a differentiation of gamonts but not of gametes.

copulate in darkness (Smith demonstrated this in 1946 for other *Chlamydomonas* species) there appears to be a difference in that the cells designated as "female"³ are at once fully able to copulate while the "male" cells show retardation in their sexual activation after a period of darkness.

Copulation in *C. eugametos* is a complex process, which consists essentially of the following phases: (a) Agglutination of sexually different gametes by the terminal parts of their flagella; (b) connection of the papillae between the flagella to a "copulation bridge"; and (c) fusion of cells.

The investigations of Förster & Wiese (34) permit a first insight into the physiology of the first of these phases. In confirmation of data obtained by Moewus (100) they could demonstrate that the gametes produce sex-specific substances. These substances cause the agglutination of gametes only after contact. However, they can be liberated into the medium under special conditions. In distinction to the mating type substances of *P. aurelia* (s. Metz, 74) they are easily separated from the cells. In this way their mode of action can be studied. When the gametes of one sex are placed into the filtrate of gametes of the other sex, isoagglutination occurs, i.e. gametes of the same sex agglutinate with one another. Since agglutination of gametes is not caused by their own substance, each kind of gamete must produce a different substance. The cells connected in isoagglutination do not form pairs but separate after a while.

Probably the terminal parts of flagella are connected with each other in isoagglutination by free groups of molecules of the substance produced by the opposite sex. Since the copulating partners also agglutinate with the terminal parts of their flagella, one can assume that these substances are also active at this location.

The two substances differ also in other properties. While the production of the "female" substance is not diminished in darkness, a detectable amount of the "male" substance is formed only on exposure to light. Therefore the readiness to react of the "male" gametes is dependent on light (see above). The two substances are also different in their dependence on other factors (temperature, pH). Chemical analysis of the "female" substance shows that it is a carbohydrate-protein complex of high molecular weight. The same may be true for the "male" substance which has not yet been investigated sufficiently.

The sex-specific substances were also detected in *Chlamydomonas reinhardtii* (35). The substances of *C. eugametos* and *C. reinhardtii* are specific for their species; filtrates of *C. reinhardtii* act only on *C. reinhardtii*, filtrates of *C. eugametos* only on *C. eugametos*. Corresponding with this species specificity, these two species could not be crossed.

Physiological differences between isogametes of the two sexes may also be detectable after copulation. Thus Lewin (68) found with *C. moewusii* that

³ The designations "female" and "male" were introduced by Moewus for his sexually interacting clones. When these were taken over by Förster and Wiese they retained the same terminology.

after copulation the flagella of one gamete continue to move while the flagella of the other become rigid. Through genetic labeling he could demonstrate that the plus gamete always behaves actively, while the minus gamete is always passive.

The first extensive genetic analysis was carried out by Lewin (70) with *C. moewusii*. Beside the sex-determining genes, the inheritance of eight alleles was investigated. Mutants were obtained following ultraviolet radiation (69); these exhibited differences which were partly morphological and behavioral, partly biochemical. After crossing two strains which are different in two pairs of alleles, the tetratypes appear regularly as zoospore- (gone-) combinations as well as the parental ditypes and nonparental ditypes. This is proof that, as in all other organisms so far investigated, the crossing-over takes place at the four strand stage.⁴ In two cases, Lewin found a low frequency of nonparental ditypes, indicating that the two genes belong to the same linkage group. In linked genes, nonparental ditypes can appear only as a result of a double four strand crossing-over.

The investigation by Lewin on *C. moewusii* was supplemented and enlarged by the work of Sager (80) on *C. reinhardi*. Sager investigated the inheritance of the sex-determining genes as well as of six others, some of which cause defects in chlorophyll synthesis. It seems certain from the high frequency of nonparental ditypes that three of the genes are not linked; the other genes may also belong to different linkage groups. So far a clear case of linkage has not been found in this species. From the frequency of tetratypes, which is a function of the second division segregation, the map distance could be calculated for all genes. It was demonstrated for one gene (Y_1) that it segregates only at the first meiotic division. At present it is not possible to decide whether this behavior depends on the gene being closely linked with the kinetochor or if it is inherited nonchromosomally.

Analysis of streptomycin resistance posed special problems (79). While this character in one case depends on a factor which shows normal mendelian inheritance, a mutant was found in which this property cannot be attributed to chromosomal inheritance. After crossing the resistant mutant with the nonresistant normal strain, all progeny are resistant, though other factors used as marker genes segregate normally 2:2. In backcrossing of the F_1 -clones with the normal strain, all F_2 -clones are also resistant when the resistant parent belongs to the plus sex and nearly always nonresistant when it belongs to the minus sex. Although the nonchromosomal manner of inheritance is clear, it is unknown how the loss of resistance is brought about.

Apart from this case, which is not yet satisfactorily explained, a hereditary behavior in accord with the theoretical expectations was established for all genes of both *Chlamydomonas* species. All pairs of alleles segregate at meiosis in the ratio 2:2. Furthermore, where more than four zoospores were formed from the zygotes, no more than four different genotypes appeared after the crossing of multiple marked strains.

⁴ Moewus found almost exclusively ditypes in *Chlamydomonas eugametos*.

Except for *Chlamydomonas*, phytomonads have not been investigated to any extent in recent years. Cave & Pocock (5) described the karyological conditions of different members of the *Volvocidae*. Starr (92) showed homothallism in *Gonium sociale*, and described its sexual reproduction.

POLYMASTIGINA⁵

In a series of papers (7 to 20) Cleveland demonstrated typical sexual processes in all species which occur in the gut of the woodfeeding roach *Cryptocercus punctulatus*. In all cases sexual reproduction is initiated by the molting hormone of the roach. However, this induction takes place in the different species at different times. Obviously each species needs a specific threshold of concentration for its activation.

At the beginning of sexual reproduction, in most species a differential division takes place, which leads to the formation of two sexually different cells or nuclei. Although the sex-determining division may coincide with meiosis (as for instance in *Urinympha*), sex-determination seems to be always phenotypical. It can take place in the haplophase or in the diplophase.

The species of the genera *Oxymonas*, *Saccinobaculus*, *Trichonympha*, *Eucomonympha*, *Leptospironympha* and *Barbulanympha* are haplonts. Therefore their sex-determination is haplophenotypical. The meiosis takes place after fertilization (zygotic meiosis). It consists of two divisions in *Trichonympha*, *Eucomonympha*, and *Barbulanympha*, and only of one single division in *Oxymonas*, *Saccinobaculus*, and *Leptospironympha*. In *Leptospironympha eupora* meiosis takes place after a cyst membrane is formed around the zygote.

The species of the genera *Notila*, *Urinympha*, *Rhynchonympha* and *Macrospironympha* are diplonts. Their sex-determination is therefore diplophenotypical. The meiosis takes place before fertilization (gametic meiosis). It consists of two divisions in *Rhynchonympha* and *Macrospironympha* and only of one division in *Notila* and *Urinympha*.

Sexual reproduction can be a gamontogamy, a gametogamy and an autogamy. In *Notila* one can speak of a gamontogamy because in this genus not haploid gametes but diploid gamonts pair with one another. These are formed by a previous differential division and for this reason they are determined already as male and female. After the fusion of the two cells the nucleus of the male gamont detaches itself from the organelles with which it is normally connected, while the nucleus of the female gamont remains connected with its organelles. Each nucleus then undergoes one division connected with chromosomal reduction (one division meiosis). This leads to the production of two male and two female pronuclei. The male pronuclei migrate to the female pronuclei, with which they fuse. In this way a "double zygote" with two syncarya is formed. This then divides into two uninuclear daughter cells through a simple cytokinesis without nuclear division.

⁵ In agreement with Reichenow (25), the reviewer considers the Hypermastigidae as a family of Polymastigina.

In species which show a fusion of true gametes (gametogamy), a differential division first takes place, in which one gamont produces two sexually different gametes. In *Trichonympha* this division occurs in the encysted stage.

All transitions from isogamety to anisogamety are found in the differentiation of gametes. While the gametes of *Oxymonas*, *Saccinobaculus* and *Leptospiromyxa* show no striking differences, there are often distinct differences of size in *Eucomonympha*, *Barbulanympha* and *Macrospiromyxa*. The most pronounced is the anisogamety in *Trichonympha*, in which the female gamete possesses a ring of granules at the posterior end surrounding a clear area which can be everted as a fertilization cone. In fertilization the male gamete, which possesses no ring of granules, adheres to the fertilization cone and penetrates the female gamete as the fertilization cone invaginates.

But also in cases where there is no morphological difference between the gametes, the sexual inequality of the copulating cells can be recognized. The nucleus of one ("male") always detaches from the organelles with which it is normally connected, and migrates to the other nucleus ("female"), which stays connected with its organelles.

This different behavior of the two pronuclei is also observed when gametes are not formed; here the sister nuclei, which are produced by a differential nuclear division, fuse with each other (autogamy). This kind of sexual reproduction takes place regularly in *Urinympha* and *Rhynchonympha*. In *Urinympha* the two sister nuclei of the one division meiosis fuse with one another. In *Rhynchonympha* a cytokinesis is combined with meiosis I; this leads to the formation of daughter cells in which the sister nuclei of meiosis II fuse. In other species (*Barbulanympha*, *Oxymonas*, *Saccinobaculus*) autogamy occurs more or less often in addition to normal gamete formation.

Hartmann (62) emphasized the importance of the discovery of a sexual inequality of autogamously fusing nuclei and of "relative sexuality" in *Trichonympha* for the theory of sexuality.

Cleveland's observations are also important for the problem of differential division, which is a general problem of differentiation. He demonstrated that the differential character of the sex-determining division in *Trichonympha* already makes itself noticeable in the chromatids. In prophase, two sets of chromatids are formed which differ in their stainability, i.e. in their different degree of spiralization. An unusual grouping and enchainment of the chromatids (end-to-end joining) makes it possible for the lighter stainable chromatids to go into the female gamete, while the darker stainable chromatids go into the male gamete. A similar process of joining which prevents random segregation of the chromatids in mitosis was observed also in *Barbulanympha*, *Rhynchonympha* and *Leptospiromyxa*.

The publications of Cleveland also contain an abundance of interesting cytological observations. Only a short summary can be given here of his observations on the structure and spiralization cycle of chromosomes in *Holomastigotoides* species (6, 21). In these species, which are especially favor-

able for cytological studies, Cleveland could demonstrate minor coils, major coils and occasionally also supercoils. The mitotic cycle of the chromosomes, he believes, depends essentially on major coils, while the minor coils never unwind. The duplication of chromosomes to chromatids also takes place in the microspiralized stage. The sister chromatids are therefore always at first connected in the manner of plectonemic coils: hence they retain for a longer or shorter time the relational coiling. This is a consequence whenever there is a duplication of chromosomes. After the loosening of the relational coiling, the sister chromatids can stay connected by pairing forces.

This latter observation provided Cleveland with a starting point for a new theory of crossing over. In a diploid strain of *Holomastigotoides diversa* he observed that a chromatid may pair partly with its sister chromatid (sister chromatid pairing), partly with the chromatid of its homologous partner (non-sister pairing). Cleveland supposes that this varied pairing tendency of a chromatid along its axis may lead to its disruption at the beginning of poleward movement and that the pieces then unite with corresponding pieces of the reciprocal chromatid. Assuming that the case of *Holomastigotoides* may be a model for crossing over in general, he believes that this occurs in the beginning of anaphase, while the chiasmata are to be regarded only as places where the chromatids change their partners. The chiasmata may lead to a crossing over but do not necessarily.

The objections which can be raised against this theory are similar to those which were raised against the concept that chiasmata are merely places where the chromatids change their partners. These objections are based on the observations which were made on unequal bivalents, on the coincidence of frequency of chiasmata and crossing over under variable physiological conditions, and on the occurrence of crossing over at complete terminalization of the chiasmata.

It seems to the author that more important than this theory is Cleveland's discovery that in meiosis of *Trichonympha* and *Barbulanympha* the tetrads can be formed by pairing of already duplicated chromosomes. For this reason Darlington's idea that chiasmata are a *sine qua non* for the formation of tetrads is wrong.

The following are perhaps the most important of Cleveland's other cytological results: (a) Meiosis may consist of one division only. In this type of meiosis the duplication of both chromosomes and kinetochores is in the one division. In contrast, in two division meiosis the duplication of kinetochores is inhibited in the first division and the duplication of chromosomes in the second. (b) There are telokinetic chromosomes in which the spindle attachment point is placed at the end. (c) There are rod-like centrioles in which the autoreproductive and the spindle producing functions are localized at opposite ends. (d) The spindle can be formed completely outside the nucleus and, by stretching, it pulls apart the chromosomes which are fixed at the nuclear membrane.

Cleveland published a number of microphotographs of the meiosis of

Barbulanympha, which show with remarkable clarity the structures of division (centrioles, spindle) and the chromosomes (spiral form, spindle attachment point) in the living cells.

AMOEBINA

Among studies with amoeba, only those of Lorch & Danielli (72, 73) can be mentioned in the scope of this review. These authors studied the effects of removal of the nucleus from *Amoeba proteus* and *Amoeba discoides* and also worked out a method for the transfer of a nucleus from one cell to another previously enucleated. A reactivation was obtained by the transfer not only between cells of the same species ("homotransfers"), but also between cells of different species ("heterotransfers"). The enucleated amoeba, which showed neither coordinated movement nor normal digestion, became fully viable again. While reactivation in homotransfers led to normal clones, in heterotransfers only occasionally was it possible to get a clone. Nevertheless it was possible to study the effect of transfer of a nucleus in reference to two characters by which the two species differ. The diameter of a nucleus in *A. proteus* is about $41\ \mu$ and in *A. discoides* about $33\ \mu$. As the measurements on the heterotransfers show, the diameter of a nucleus is regulated largely in accordance with the cytoplasm to which it is transferred. Nuclei of *A. proteus* become smaller after transfer into the plasm of *A. discoides*, and nuclei of *A. discoides* become larger in the plasm of *A. proteus*. Shortly after transfer the influence of the nucleus on the shape of the cell makes itself noticeable; however this effect disappears completely after a few days. Then the cell form is restored in accordance with its cytoplasm. In both cases the influence of cytoplasm is dominant.

RADIOLARIA

The development of the Radiolaria is one of the unsolved problems of Protozoology (100). From Chatton's studies, which are supplemented by recent observations (63) it appears that "anisoprogenesis," a process of swarmer formation described by authors in the past, does not belong to the life cycle of the Radiolaria: the "anisospores" are swarmers of parasitic dinoflagellates.

On the contrary the "isospores" which were observed again by Le Calvez (1935) in *Coelodendrum ramosissimum*, are considered as true swarmers of the Radiolaria. According to the earlier investigations the formation of isospores, which in most cases are equipped with a crystal and therefore designated as "*Kristallschwärmer*," is connected with peculiar cytological processes. Many Radiolaria possess a very large nucleus (primary nucleus). This nucleus breaks down shortly before swarmer formation into many small nuclei (secondary nuclei) which then become the nuclei of the swarmers. These observations led Hartmann (61) to the assumption that such primary nuclei are "polyenergid" and comparable with the macronuclei of the ciliates, i.e. composed of numerous separate genomes which at swarmer formation break down through a "multiple division" of the nucleus.

Recently Hollande & Enjume (64) by cytological studies of *Thalassicolla* which multiplies only by swarmer formation, could actually confirm that the primary nucleus becomes polyploid by a process of consecutive intranuclear mitoses. Grell (53), working with *Aulacantha scolymantha* (a species with over 1000 chromosomes), found evidence that the polyploidy is not a permanent condition but the result of a long lasting growth period. In *Aulacantha* the polyploidy seems not to be the result of intranuclear mitoses but of endomitotic divisions of the chromosomes. Some observations suggest that the chromosomes, which do not show distinct differences in size and shape, correspond to whole genomes. However all these interpretations will be hypothetical as long as no details are known about the course of the development and especially about the disintegration of the primary nucleus in the process of swarmer formation. The bipartition of the nucleus of *Aulacantha*, which proceeds without a definitely recognizable spindle apparatus, is described in detail by Grell with the aid of microphotographs taken *in vivo*.

FORAMINIFERA

Investigations with Foraminifera in recent years have led to new and surprising results. It had been believed that the whole development takes place in diplophase until Le Calvez (66) in a fundamental investigation, obtained results, which indicates that the alternation of generations is heterophasic. The asexual (before described as microspheric) generation is diploid, the sexual (before described as macrospheric) generation is haploid. Meiosis takes place in the agamonts (called "schizonts" by Le Calvez) shortly before formation of agametes (= gamonts). It is therefore neither zygotic nor gametic but intermediate. The Foraminifera occupy a unique position in the animal kingdom by possessing a heterophasic life-cycle, which otherwise occurs only in the plant kingdom.

That meiosis in Foraminifera takes place before formation of the agametes was detected by Le Calvez only in two species, *Patellina corrugata* and *Discorbis vilardeboana*, but Grell observed the same in *Myxotheca arenilega* (56) and *Rotaliella heterocaryotica* (57, 58). Both generations, the agamont that multiplies asexually and the gamont that reproduces sexually, can be alike morphologically (homomorphism) and different only in size. This condition is shown by all monothalamous genera thus far investigated (*Allogromia*, *Myxotheca*, *Iridia*), and also by the small polythalamous species *R. heterocaryotica*. In the majority of the Foraminifera, however, the two generations are different morphologically (heteromorphism). The difference in the initial chamber, which led to the terms "microspherical" and "macrospherical," may not be the only one. In some cases the two generations are so different that they were described as separate species by systematists, as Le Calvez (67) demonstrated for *Discorbis patelliformis* (Brady) and *Discorbis erecta* (Sidebottom).

The few species which have been investigated more carefully show clearly that the Foraminifera represent a group of Protozoa whose reproduction ex-

hibits much greater variety than was assumed until now. It is a pity that the investigation of this group has been conducted nearly exclusively from the taxonomic point of view.

In many species the gamonts unite into pairs (gamontogamy). They surround themselves with a cyst composed of small particles of detritus (*Patellina*, *Spirillina*) or the two shells are joined by means of a cement substance. The gametes then copulate in the enclosed area formed by the gamonts. They are in most cases ameboid. Only in species of *Discorbis* do they possess three flagella and move freely (66).

Le Calvez demonstrated that in *Discorbis mediterraneensis* the gamonts are differentiated sexually. If two gamonts are placed in a dish, they either creep towards one another or behave indifferently. He put together in all possible combinations all the gamonts, which descended from the same agamont. From the behavior it was clear that there must be two groups and therefore two different sexes.

On the basis of his observations in *D. mediterraneensis* Le Calvez (50) assumes that sex-determination in all Foraminifera takes place at meiosis and is therefore genotypical. Even in *D. mediterraneensis* this is not conclusively established and the generalization is certainly not admissible. Subsequently, a phenotypical sex-determination has been indicated in many species.

In *M. arenilega*, which is monothalamous, the gamonts do not pair but instead each gamont releases free-swimming gametes into the sea water. If a single gamont is isolated in a dish filled with sea water before the formation of gametes, the gametes may copulate with one another. Thus *M. arenilega* is a homothallic species with phenotypic sex-determination (56).

Besides this, it appears that autogamy is not rare in Foraminifera. The first case of autogamy was observed by Arnold (1) in *Allogromia laticollaris*. Sexual reproduction in *R. heterocaryotica* also leads to autogamy (58). Since this species could be cultivated easily in pure culture, the whole development could be clarified (Fig. 1). When a gamont which contains only one nucleus in its initial chamber [1] reaches a certain size, many nuclear divisions take place which lead to the production of the gamete nuclei. For these nuclear divisions it is typical that all, except the last one, proceed asynchronously. The last mitosis occurs synchronously [2]. Grell assumes, therefore, that the last mitosis has the character of a differential nuclear division, being combined with sex-determination. However, the resulting gametes do not show any morphological differences. They are ameboid and fuse autogamously within the same gamont [3, 4]. Before the young agamonts, which derive from the zygotes, leave the gamont, two nuclear divisions take place and in this way four daughter nuclei are formed in each agamont [5, 6]. In the beginning all four nuclei are equal; however, after a short time one of them swells up and develops a nucleolus while the three others remain condensed. The meaning of this nuclear differentiation is understandable in considering the further development. While the larger nucleus moves into one of the younger chambers, the three smaller remain in the initial chamber [7].

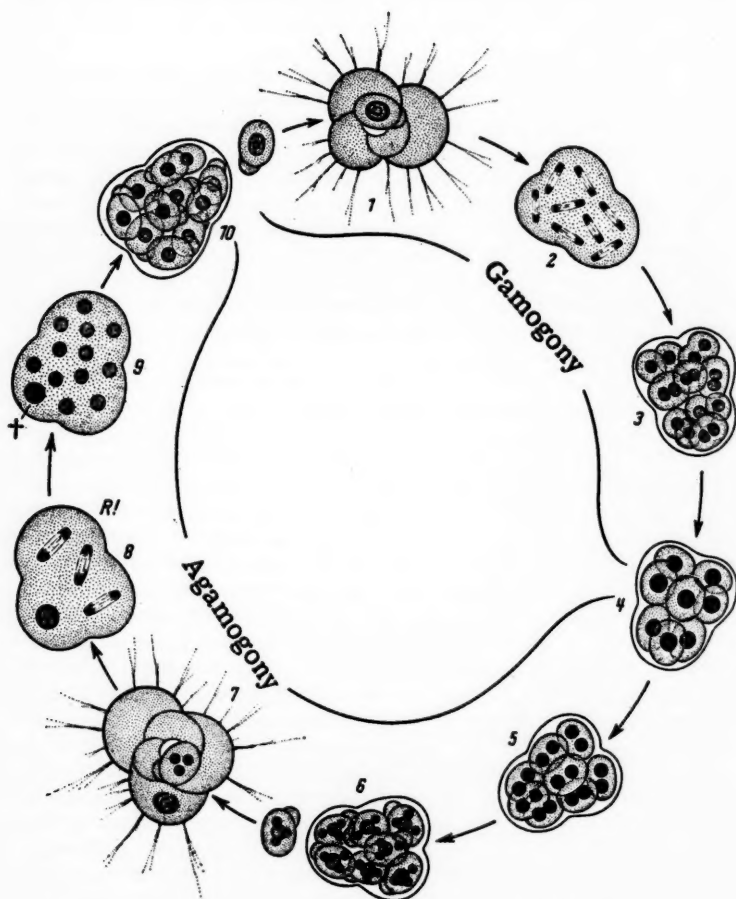


FIG. 1. The cycle of development of the foraminiferan *Rotaliella heterocaryotica*. 1 Gamont. 2 Last nuclear division of gametogenesis. 3 Copulation of gametes within the shell of the gamont (autogamy). 4 Zygotes. 5 Binucleate young agamonts. 6 Tetra-nucleate young agamonts. 7 Adult agamont. 8 First meiotic division of the generative nuclei. 9 End of the second meiotic division (degeneration of the somatic nucleus). 10 Agamont with twelve agametes (=young gamonts). R! Meiosis. After Grell (58).

This process takes place during the growth of the agamont. In meiosis which consists of two divisions, only the smaller nuclei participate [8, 10]. The larger nucleus becomes pycnotic, and at the end of meiosis it is dissolved in the cytoplasm or expelled from the cell. The smaller nuclei may be designated therefore as generative, the larger nucleus, which seems to regulate metabolism during the growth period, as somatic. *R. heterocaryotica* presents the first case of a nuclear dualism outside the ciliates. Meanwhile, more cases of autogamy and nuclear dualism have been found in Foraminifera (Grell, unpublished).

COCCIDIA

While the mode of alternation between haplophase and diplophase in Foraminifera had not been explained until recent years, it had long been known that the coccidia are haplonts with zygotic meiosis. In most species of Coccidia the sexual reproduction (gamogony) alternates with two different asexual processes (schizogony, sporogony).

However in some species of Coccidia no schizogony has been found. Observations of Ganapati (36) in *Myriospora polydora*, a parasite in the polychaete *Polydora ciliata*, show that the earliest stages which he called "merozoites" grow up directly into the sexually differentiated forms (gamonts). Some of them develop into microgamonts, which break down into "microgametoblasts" each of which yields about 12 comma-shaped microgametes. The others grow into large macrogamonts. Whether schizogony occurs in another host, or is entirely absent from the life cycle, is not known.

Grell (54) demonstrated with certainty that schizogony does not occur in *Eucoccidium dinophili* which could be found in the body cavity of *Dinophilus gyrociliatus*, a marine archiannelid. The sporozoites develop immediately into gamonts. For this reason Grell assumes that schizogony may also be missing from the life cycle of other Coccidia in which it has not been observed. He suggests a classification of the Coccidia, similar to that of the gregarines: suborder *Eucoccidia* (extracellular development, without schizogony) and suborder *Schizococcidia* (intracellular development, with schizogony).

In *E. dinophili* the development proceeds as represented in Figure 2. The sporozoites [1] penetrate the gut wall and enter the body cavity [2] where they develop either into large elongated macrogamonts [8] or into small round microgamonts. By multiple fission the microgamonts form 12 to 32 microgametes [4 to 6]. These are bowl-shaped and possess two flagella of unequal length [7]. After fertilization [9] the zygote surrounds itself with an oocyst membrane [10]. After that, meiosis and sporogony take place leading to the formation of many spores [11 to 13]. The oocyst bursts open when the spores still possess only one nucleus. The spores lie free in the body cavity of the host. In most cases six sporozoites are formed in each spore.

The question of sex-determination could be answered in this species [see discussion of Wenrich (99, p. 203)] which can be easily cultivated together

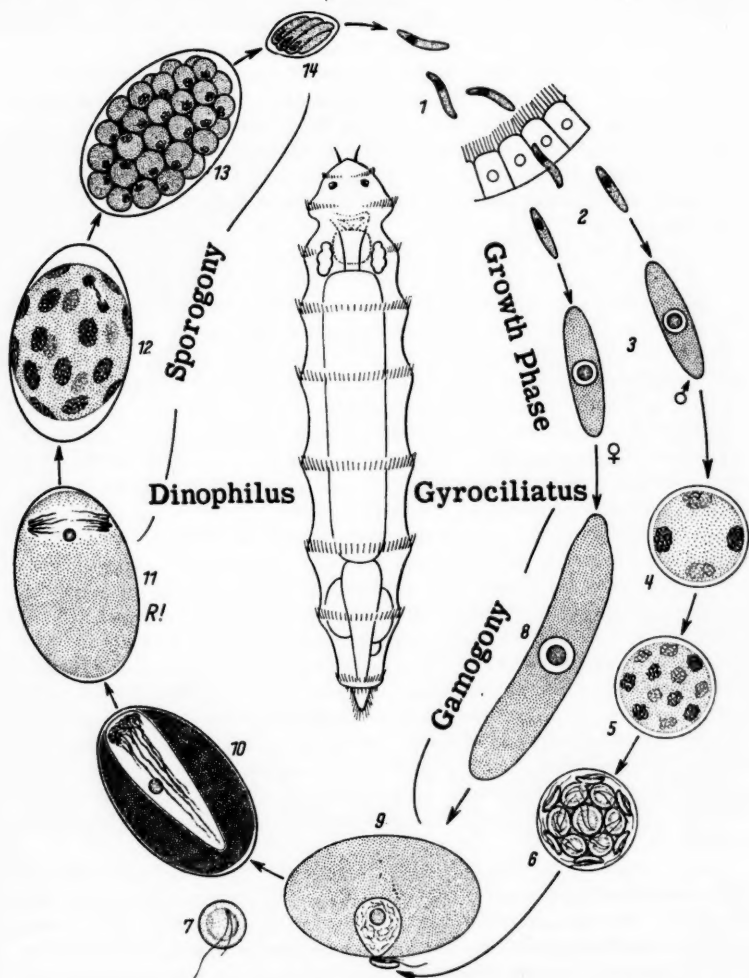


FIG. 2. The cycle of development of the coccidian *Eucoccidium dinophilus* in the body cavity of the marine archiannelid *Dinophilus gyrocilatus*. 1, 2 Sporozoites, penetrating the gut wall. 3 Young gamonts. 4-6 Microgamont in gamete formation. 7 Single microgamete. 8 Macrogamete. 9 Fertilization of the macrogamete. 10 Zygote. 11 First nuclear division of sporogony (meiosis). 12 Later nuclear divisions. 13 Formation of spores. 14 Single spore with sporozoites. After Grell (54).

with its host in a pure culture. If sex-determination were genotypic, the sex-determining genes would segregate in meiosis and spores should be formed which would belong either to one or to the other sex. Hence from one spore both sexes could not arise. After infecting the worms with one spore only, all sporozoites develop to macrogamonts. Microgamonts never arise in the first generation of a single spore infection. The macrogamonts develop further parthenogenetically. If a mass infection is carried out using the spores developed parthenogenetically from a single spore only, one obtains both macrogamonts and microgamonts. It can be concluded from this that sex-determination is phenotypic. In an infection with only one or a few spores only macrogamonts are formed, while in a heavy infection the conditions within the body cavity of the host are adjusted so as to make possible the development of microgamonts too. The sporozoites in the spore are not yet sexually determined. Sex-determination takes place after the sporozoites have penetrated the body cavity.

By x-ray treatment of the spores in *E. dinophili*, chromosomal breakages can easily be produced which, on account of bridge formation in consecutive mitoses, change the normal course of sporogony (59).

CILIATA

Cytological investigations published since the appearance of Sonneborn's report in the *Annual Review of Microbiology* (1949), are concerned primarily with the structure of the macronucleus. The capability to regenerate and to divide unequally suggests that the macronucleus is of polyploid and polyploid character (30, 55). Sonneborn states that the ploidy-level for the macronucleus of *P. aurelia* is between 80 and 300. In what way this polyploidy is brought about is now a question.

In the suctorian *Ephelota gemmipara*, the conjugation of which has been studied by Grell (51), the development of the macronuclear anlage lasts several days. In the young macronuclear anlage the chromosomes can be recognized as distinct thin threads. While the macronuclear anlage swells up, the chromosomes divide first in two, then into four and finally into eight chromatids. There is a probability that more divisions occur. From this observation it is clear that polyploidization is brought about endomitotically. Later the macronuclear anlage assumes the structure of an interphase nucleus, i.e. the chromosomes despiralize and fill out the whole macronuclear anlage which becomes weakly stainable. Only a small region, regarded as a "compound chromocenter" remains intensively feulgen positive; however this interphase stage is only of short duration. While assuming its definite form, the macronucleus becomes more and more feulgen positive. The completely developed nucleus shows a thread-like structure. Grell supposes that the threads are the chromosomes.

Also in the suctorian *Tokophrya* species (52) the chromosomes can be recognized in the macronucleus as weakly spirialized threads. They can even be photographed *in vivo*. Therefore in its structural aspect the macronucleus

represents a type of nucleus similar to that of the dinoflagellates: its chromosomes are always present as threads rich in deoxyribonucleic acid.

Phases indicative of endomitotic polyploidization have not been observed in other cases. In *Paramecium bursaria* Egelhaaf (26) demonstrated that the telophase nucleus, which has been determined as macronuclear anlage, immediately passes over into the interphase stage. In the beginning of this process parts of the chromosomes retain their stainability, thus forming a sort of "compound chromocenter" within the macronuclear anlage. Later this "chromocenter" also disappears in the achromatic interphase nucleus. By continuous formation of DNA, the nucleus changes to the feulgen positive final stage of its development.

The lack of a recognizable endomitotic period does not mean, as Egelhaaf also emphasized, that it does not take place. It is possible that the endomitotic divisions are shifted into the interphase stage in which they cannot be seen on account of the despiralization of the chromosomes. Such "crypto-endomitoses" are known also from the interphase nuclei of higher animals and plants.

The differentiation of a micronucleus and a macronuclear anlage following conjugation is determined by extranuclear factors. Nanney (75), by use of centrifugation experiments, showed that in *Tetrahymena* the nuclei which normally become micronuclei can develop into macronuclear anlagen, while prospective macronuclear anlagen can become micronuclei. It was found by Egelhaaf (26) that in *P. bursaria* after the third metagamous division, which is followed normally by differentiation of a micronucleus and a macronuclear anlage, the nuclei can still be redetermined. What extranuclear factors cause the determination is as yet not clear. In all probability they are to be found in regional differences of the cytoplasm as was indicated by Sonneborn (83).

In connection with this question, a report of Seshachar & Dass (82) is of interest. They observed in *Epistylis articulata* that the micronucleus of individuals, which had lost their macronucleus as a result of an abnormal vegetative division, may divide like a synkaryon, forming a new micronucleus and a new macronuclear anlage.

In the multinucleate ciliate *Loxodes rostrum* Fauré-Fremiet (32) confirmed the concept of Bütschli, that macronuclei never divide during vegetative development but are continuously formed by micronuclei. In *Centrophorella fistulosa* no sign of nuclear division at all could be detected by Fauré-Fremiet. He supposes that during the "interdivisional period," individual nuclei become endomitotically polyploid and then as "polyenergid nuclei" break down into a number of subnuclei. "Among these some evolve in the somatic and macronuclear direction, before disappearance by karyolysis; others corresponding to the micronuclei grow by endomitosis and reconstitute the polyploid and polyenergid nuclei."

The division of some of the Oligotricha was investigated thoroughly by Fauré-Fremiet (29). He also described in detail the division of *Urocentrum turbo* where the behavior of the kinetosomes is especially considered (31).

The conjugation of *Epistylis articulata* was described by Dass (23) and that of *Spirochona gemmipara* by Tuffrau (98).

Corliss (22) demonstrated that in the cysts of *Tetrahymena rostrata* an autogamy takes place regularly. Autogamy in *Paramecium polycaryum* was described by Diller (24). In this species conjugation has not been observed.

A detailed summary by Beale (2), containing many unpublished data, deals with the genetics of *P. aurelia*. Therefore, only publications that have appeared since will be treated here.*

Sonneborn (85, 86) isolated a mutant in which an unequal distribution of nuclei in cell-division takes place continuously in a high percentage of the animals. Cells without macronuclei arise in this way. Frequently a daughter cell of an animal, which passed through an autogamy, contains no macronuclear anlage. Then a macronucleus may grow up from a fragment of the old one (macronuclear regeneration). Genetical analysis showed that this character (unequal nuclear distribution) is determined by a single recessive gene *am*. Because it is easy to obtain macronuclear regeneration (without temperature treatment) in this mutant, it was used by Sonneborn to construct "heterocaryotic paramecia," i.e. cells which possess a different genotype in micronucleus and macronucleus. In this way, the result of a previous experiment of Sonneborn (101), concerning which there were some objections in respect to the methods used, was confirmed: the K-gene responsible for the killer-character is not active in the micronucleus, even when it is present homozygotically. It is suggested that this genetic inactivity of the micronucleus applies also to other genes.

The investigations on the problem of senescence and rejuvenescence with which Sonneborn and his associates (84, 87) are concerned can not be properly discussed here because they are not yet concluded.

Butzel (4) made an analysis of two mutants of variety 1 of *P. aurelia* which, as in the well known one-type stocks, manifest only mating type I. This investigator also found a modifying factor which determines the frequency of mating type I in two-type stocks. He developed a hypothesis which traces the determination of mating types to the activation of mating type genes.

In recent years it has become possible to use yet another ciliate, *Tetrahymena pyriformis*, for genetical analysis. This species differs from *P. aurelia* in possessing a multiple mating type system (27, 28). It corresponds to *P. aurelia* in the manner of mating type determination, which takes place during the development of the new macronuclear anlage in the daughter cells of the exconjugants (76).

In one variety (Number 1) of *T. pyriformis*, seven mating types have been found. Besides the normal strain, which manifests all seven mating types, Nanney, Caughey & Tefankjian (78) detected two mutants. One of the

* The papers of Sonneborn and his associates dealing with serotype inheritance in *Paramecium aurelia* are not treated in this review.

mutants, designated as "family A," does not form the mating types IV and VII, the other, designated as "family B," does not produce mating type I. If the families A and B are crossed, the F_1 -generation forms all seven mating types. In the F_2 -generation a segregation into three classes takes place, one of which corresponds to family A, a second to the F_1 -generation and the third to family B. The simplest interpretation of the crossing results would be to assume that the difference between the two mutants is due to one pair of alleles $mt^{(I)-}$ and $mt^{(IV,VII)-}$. However other interpretations, involving two or several closely linked genes, cannot be fully excluded. The most important result of these investigations is the finding that the spectra of mating type potentialities, typical for the different strains, are fixed genetically; the determination of the particular mating type may be due to the activation of a corresponding mating type gene. This activation, however, is caused by non-genetic factors.

DIATOMS

Our knowledge of the reproduction of diatoms has been greatly advanced in recent years. The most important advance is the demonstration that the members of both Centrales and Pennales are diplonts, meiosis taking place during the formation of gametes. In spite of this conformity there are fundamental differences between the two groups in the mode of gamete differentiation and in the manner of mating. While the interpretation of sexual differentiation and sex-determination is not difficult in Centrales, it is very problematic in Pennales.

Centrales.—The reproduction of Centrales was misinterpreted for a long time. It was believed until recently that auxospore formation had no connection with sexual reproduction, though the observations of Iyengar & Subrahmanyam (65) in *Cyclotella meneghiniana* had indicated that the auxospores develop from zygotes. Supposedly in this case karyogamy takes place through the autogamous fusion of two gone nuclei. Later Stosch (94) demonstrated oogamety satisfactorily for the first time in *Melosira varians*. After some preceding divisions "oogonia" and "spermatogonia" are formed in the filaments of *M. varians*. Meiosis is gametic and consists of two steps. In the oogonia no cell divisions are connected with the nuclear divisions. In each divisional step one of the daughter nuclei becomes pycnotic and consequently only one gone nucleus remains. For this reason, only one egg cell arises from each oogonium. In spermatogonia, however, nuclear divisions are connected with cell divisions so that all four gones become "sperms." Nevertheless in this process a part of the spermatogonium mother cell is left over as a "remaining body." The sperms are flagellated. After fertilization the zygote develops into an auxospore. A similar type of sexual reproduction was described by Geitler (40) for *Cyclotella tenuistriata*; however, fertilization in this species takes place earlier than in *M. varians*, occurring when the nucleus of the oogonium is at the diplotene stage.

Stosch (93, 95) also detected oogamety in many marine Centrales. In the

species *Biddulphia mobiliensis*, in which Bergon described "microspore formation" for the first time, Stosch demonstrated that the "microspores" are actually sperms and that the "auxospore mother cell" (=egg cell) can develop only after being fertilized by one of those sperms. In this case numerous biflagellated sperms are developed in each "microsporangium" since meiosis is preceded by a number of ordinary cell divisions through which the spermatogonia multiply.

Oogamety is common to all Centrales which have been closely investigated though some species differ remarkably in the manner of egg- and sperm-formation. For this reason there is no doubt that in Centrales the haplophase is sexually differentiated. But the sex-determination takes place in the diplophase and it is phenotypical. In the filamentous *M. varians* it can be observed that oogonia and spermatogonia are produced on the same filament. In other species, the monoecious character of the diplophase could be detected by the use of clone cultures.

Pennales.—Unlike the Centrales which form free swimming sperms that leave the mother cell in order to find an egg cell (oogamety), the gametes of Pennales are always morphologically equal (isogamety). Besides this the mother cells of gametes (gamonts) come together in pairs (gamontogamy). Therefore sexual reproduction in Pennales resembles that of the ciliates. In both groups, after mating, meiosis takes place in each gamont. It consists of two steps and leads to the formation of four gone nuclei. While in the ciliates three of these four nuclei degenerate and the remaining one undergoes a third (postmeiotic) division which produces the two pronuclei (migratory and stationary nucleus), in Pennales the four gone nuclei are the gamete nuclei. In ciliates only gamete nuclei are formed but in Pennales true gametes are produced.

By degeneration of some of the gone nuclei less than four gametes are formed in the majority of Pennales. The formation of four gametes can be regarded as the original type of gamete formation in Pennales. Pascher described such a case in 1932, but its validity is uncertain since the copulation of the gametes was not seen.

Investigations in recent years show that a great variability exists in gamete production. Essentially two possibilities may be distinguished:

(a) Each gamont forms two gametes. Since the cell division which leads to the formation of the two gametes takes place after the first meiotic division, each of the two gametes gets two gone nuclei. The time of degeneration of one of these nuclei varies with particular species. The most primitive condition is found in *Navicula radiosa* (44) where the degeneration takes place quite late. Here the gametes mate in the binucleated state. Two pairs of pronuclei are present even in the zygotes. Not until the auxospore is growing does one pair of nuclei degenerate. In other species the degeneration of the supernumerary gone nuclei takes place in the gametes, as for instance in *Gomphonema* species (42), *Navicula halophila* (96) and *Rhoicosphenia curvata*.

(b) Each gamont forms only one gamete. This possibility is realized in the most primitive manner in *Eunotia* (38, 39) and *Cocconeis* species (41), because in these species one cell division still takes place in each gamont. This division, however, is unequal and leads to the formation of a large cell which becomes the gamete and a small cell which aborts. In both genera this differential division is preceded by a stratification of the cell content which fixes the position of the spindle. In *Eunotia* the gamete is always formed on the side of the under valve (hypotheca), in *Cocconeis* on the side of the upper valve (epitheca).

In other species no cell division takes place but the whole content of the gamont changes into one gamete. It may be worth mentioning that in *Navicula cryptocephala* two gone nuclei remain preserved within each gamete, so that two pairs of pronuclei are present in the zygote (compare with *N. radiosa*). The degeneration of the supernumerary pair of nuclei takes place when the auxospore grows up.

While in most Pennales allogamy prevails, i.e. fusion occurs only between gametes originating from different gamonts, some cases of autogamy have been reported. An incidental fusion of the two gametes originating from the same gamont occurs in *Synedra ulna* (49). In a race of *Gomphonema constrictum* var. *capitata* (42) this process seems to be the rule. In *Denticula tenuis* (46) no gametes are formed; instead the sister nuclei of the same cell fuse to form the synkaryon.

In allogamous species the gamonts usually form a special "copulation jelly" after mating. The gametes either move directly to the partner or they use special copulation tubes to reach it. In the latter case generally one copulation tube is formed, but in *Amphipleura pellucida* (43) two tubes are observed. In some cases the gametes of both gamonts meet each other "half way" and behave alike ("physiological isogamety"). In other cases each gamont forms a "migratory gamete" and a "stationary gamete" ("physiological anisogamety"). The migratory gamete of a gamont reaches the other gamont directly (*Navicula*, *Gomphonema*, *Cocconeis* species) or by means of a copulation-tube (*Nitzschia* species), to fuse with the stationary gamete. In *A. pellucida* the migratory gametes move in two copulation tubes in opposite directions at the same time. As in ciliates in all these cases of "physiological anisogamety" a reciprocal fertilization takes place.

Finally there is still the possibility that one gamont forms two migratory gametes and the other two stationary gametes [*S. ulna*, Geitler 1939; *N. halophila* (97)]. Even in those cases of gamont pairing, where each of them produces one migratory and one stationary gamete, constant differences can be recognized between the behavior of the two gamonts. As early as 1932 Geitler showed in *Gomphonema parvulum* that the gamont which forms a "jelly cushion" before pairing, is always the passive partner.

If one agrees with Hartmann (63) that each fusion of cells or nuclei which is followed by karyogamy presupposes a sexual difference between the interacting cells or nuclei, then the same difficulties will arise in Pennales as in cili-

ates. On one hand there are facts, indicating a bipolarity of gamonts (anisogamonty) as was shown by the cases cited in the previous section. If the gamonts are sexually different, then the sex-determination should be diplophenotypical because the gamonts mate also in clones. On the other hand the sporadic cases of autogamy and the different behavior of "migratory" and "stationary" gametes indicates that the gametes of the same gamont may be different sexually. The fact that these gametes do not usually mate may be interpreted on the basis of "self sterility." In the case of a sexual difference between the gametes produced by the same gamont, the sex-determination may be connected with meiosis, but might be genotypical or phenotypical. The problem culminates in the case of *S. ulna*, because here two gamonts fuse, one of which forms always only migratory, the other only stationary gametes. In addition, it was observed that sometimes the gametes of the same gamont fuse autogamously. One can also surmise that gamonts as well as gametes are sexually different and the two "levels of differentiation" do not need to coincide. At present the question of sexual differentiation of Pennales, which is discussed in detail in the papers of Geitler (37, 46), is not answered clearly.

It may be added that two metagamous mitoses seem to take place regularly in the auxospore of Pennales (47) and Centrales (40). These are not connected with cell division. It is characteristic of them to yield each time one abortive sister nucleus.

CONJUGATAE

Our knowledge of this group has increased recently. After it became possible to raise these algae in pure culture, Starr (89) succeeded for the first time in inducing sexual reproduction. If clones of *Cosmarium botrytis* var. *subtimidum* which are isolated from their natural habitat are mixed, they can form zygotes only in certain combinations. Thus this species is dioecious (heterothallic). In other varieties of the same species, as well as those of *Cosmarium subcostatum*, it was possible to isolate dioecious clones while one clone of *Closterium parvulum* (91) proved to be monoecious (homothallic).

By mixing clones together the process of gamete fusion and zygote differentiation can be induced at will in *C. botrytis* var. *subtimidum*. It was also possible to initiate the germination of zygotes. All these processes were photographed (90). After the protoplast is hatched from the zygote membrane, the synkaryon divides twice. The meiotic character of these nuclear divisions can be easily recognized. A cell division is combined only with meiosis I. In each daughter protoplast one of the two sister nuclei of the second meiotic division degenerates.

The zygotic meiosis also could be shown genetically by the demonstration of haploid segregation (88). The two genes which originate from the same zygote are as a rule of different sex. Therefore sex-determination is haplogenotypical. The segregation of the sex-determining genes usually takes place in the first meiotic division. In addition the inheritance of a

lethal factor was investigated. This factor had no effect in the haploid phase, but in homozygotic constitution of the diploid zygote it caused death. It is not linked with the sex-determining gene and segregates in the first meiotic division.

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METABOLISM OF NITROGENOUS COMPOUNDS^{1,2}

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Interests of the writer and limitations of space have determined the scope of this review. It deals mostly with amino acids. Only a few references to studies on purines, pyrimidines and related compounds have been cited, and no works on metabolism of inorganic nitrogen compounds have been included. In some instances backgrounds of problems have been summarized in order to clarify the significance of recent developments. Of course, not all relevant papers that appeared during 1955 could be included in the discussions of the selected topics. An attempt has been made to mention those that are most pertinent, but very likely many important ones were overlooked; other omissions were demanded by a lack of space.

Recent publications of general interest include the following: "A Symposium on Amino Acid Metabolism" (1); the reviews on "Transamination" by Meister (2) and "Intermediates in Amino Acid Biosynthesis" by Davis (3); "Studies of Biosynthesis in *Escherichia coli*" by Roberts *et al.* (4), and the chapters on "Nonoxidative, Nonproteolytic Enzymes" by Axelrod (5), "Metabolism of Amino Acids and Proteins" by Ehrensverd (6), and "Nucleic Acids" by Brown & Todd (7).

DECARBOXYLATION OF AMINO ACIDS

Sher & Mallette (8, 9) prepared lysine and arginine decarboxylases from *Escherichia coli* and completely separated the two enzymes for the first time by a series of ammonium sulfate fractionations. To free the arginine decarboxylase preparations from last traces of lysine decarboxylase, advantage was taken of the greater stability of the former at pH 4.4.

Cell suspensions of *E. coli* and *Aerobacter aerogenes* contained decarboxylases for diaminopimelic acid and lysine and converted diaminopimelic acid stepwise to lysine and cadaverine [Dewey, Hoare & Work (10)]. Diaminopimelic acid decarboxylase was prepared in cell-free form and separated from lysine decarboxylase by fractional extraction of *A. aerogenes* with acetone. Denman, Hoare & Work (11) studied the effect of pyridoxine deficiency on cellular levels of diaminopimelic acid and lysine decarboxylases in a mutant of *E. coli* which required pyridoxine. When lysine was omitted from the growth medium, lysine decarboxylase was not found in the cells. The apo-

¹ The survey of literature for this review was concluded in December, 1955.

² The following abbreviations are used: ADP (adenosine diphosphate); ATP (adenosine triphosphate); DPN (diphosphopyridine nucleotide); DPNH (reduced diphosphopyridine nucleotide); IAP (imidazoleacetol phosphate); IGP (imidazole-glycerol phosphate); TPN (triphosphopyridine nucleotide); TPNH (reduced triphosphopyridine nucleotide); CoA for coenzyme A.

enzyme of diaminopimelic acid decarboxylase was about twice as high in pyridoxine-deficient cells grown without lysine as in control cells or pyridoxine-deficient cells grown with lysine. The authors concluded that the main function of diaminopimelic acid decarboxylase is that of providing lysine for the cells.

Virtanen & Hietala (12) reported that *E. coli* decarboxylated γ -hydroxyglutamic acid to α -hydroxy- γ -aminobutyric acid. It would be interesting to know whether the responsible enzyme is the same one, found by Umbreit & Heneage (13) in *E. coli*, which decarboxylates β -hydroxyglutamic acid to γ -amino- β -hydroxybutyric acid and which differs from glutamic acid decarboxylase.

Kauffmann & Moller (14) tested more than 400 *Salmonella* cultures for amino acid decarboxylases. They usually found decarboxylases for lysine, arginine and ornithine, but not for glutamic acid.

Hurwitz (15) studied the enzymatic phosphorylation of 11 analogs of vitamin B₆ and tested their effect on tyrosine decarboxylase from *Streptococcus faecalis*. Under conditions in which the concentration of pyridoxal phosphate did not saturate tyrosine apodecarboxylase, a 45,000:1 molar ratio of unphosphorylated analog to pyridoxal phosphate was not inhibitory in the decarboxylase reaction. When certain of the analogs, e.g., deoxypyridoxine, 4,5-dihydroxymethyl-2-methylpyridine, 3-amino-4,5-dihydroxymethyl-2-methylpyridine and pyridoxine, were preincubated with ATP and a tyrosine apodecarboxylase preparation which contained kinase capable of phosphorylating pyridoxal, the decarboxylase reaction was inhibited. After incubation with ATP and the kinase, none of the compounds could replace pyridoxal phosphate in activating the decarboxylase.

DEAMINATION

Work (16) investigated the action of L-amino acid oxidases on the optical isomers of diaminopimelic acid. Oxidase from *Neurospora* attacked *meso*- and L-L-diaminopimelic acid, L-diaminopimelic acid-D-monoamide, L-lysine and L-methionine at similar rates. Two atoms of oxygen per mole of the L-L-isomer of diaminopimelic acid and one atom per mole of the *meso* isomer were consumed. Both isomers were oxidized very slowly by snake venom oxidases. D-D-Diaminopimelic acid was not oxidized by any of the oxidases.

Wiame & Pierard (17) reported that a cell-free extract of *Bacillus subtilis* contained a DPN-linked dehydrogenase for L-alanine. The action of the enzyme was analogous to that of glutamic acid dehydrogenase, and pyruvic acid, ammonia and DPNH were the end products. The dehydrogenase was not active on the D-isomer.

Evidence that serine and threonine deaminases are separate enzymes was obtained in experiments by Boyd & Lichstein (18). The ratios of the two activities, with the DL-isomers as substrates, varied in several organisms and were affected by nutritional factors. Pardee & Prestidge (19) studied in-

duced formation of serine and threonine deaminases in *E. coli* and concluded that the same enzyme deaminates D-serine and D-threonine but that L-serine deaminase and L-threonine deaminase are separate enzymes. Kristof-fersen & Nelson (20) found that *Lactobacillus casei* released ammonia from serine but not from phosphoserine. From their studies the authors suggested that two serine deaminases with different pH optima might be present.

By grinding cells with alumina, Metaxas & Delwiche (21) were able to separate L-cysteine desulfhydrase of *E. coli* from its coenzyme, pyridoxal phosphate. The enzyme was not affected by adenosine-5-phosphate or biotin, but α -ketoglutarate, tryptophan, glutamic acid, alanine and tris-(hydroxy-methyl)-aminomethane were inhibitory. Artman, Markenson & Ölitzki (22) reported that the cysteine desulfhydrase in cell-free extracts of *E. coli* was inhibited irreversibly by streptomycin. Williams & McIntyre purified aspartase (23) and adenosine deaminase (24) from *Bacterium cadaveris* and concluded that the two enzyme systems are unrelated.

D-AMINO ACIDS

In the minds of microbiologists D-amino acids are continuing to progress from the category of "unnatural and unusual" to the status of "metabolically active and of widespread occurrence." Specific D-amino acids are mentioned in other places in this chapter as they fit in with the subject under discussion.

Stevens, Gigger & Bowne (25) examined hydrolysates of dried cells of *Bacillus brevis* for D-amino acids and found approximately equimolar quantities of D-phenylalanine and D-aspartic acid amounting to more than 10 per cent of the total α -amino acid nitrogen. These two D-amino acids occurred in the cells mainly in combined form, extractable with hot ethanol or dilute alkali at room temperature. Approximately 15 per cent of the extracted D-amino acid nitrogen was dialyzable. Hydrolysates of the dialyzed material contained approximately equimolar amounts of L- and D-aspartic acids, L- and D-phenylalanine, L-threonine and a smaller amount of L-serine.

Up to 40 per cent of the D-alanine in *S. faecalis* occurred as one or more unidentified derivatives, extractable by hot trichloroacetic acid; the remainder was firmly bound in the insoluble portions of the cell wall and liberated by acid hydrolysis [Snell, Radin & Ikawa (26)]. As much as 14 per cent of the total nitrogen of the cell wall occurred as D-alanine. Large amounts of L-alanine were also present. When D-alanine of the growth medium was replaced by D- α -amino-butyric acid, cells contained no D-alanine but D- α -aminobutyric acid was found, although in amounts less than the amount of D-alanine normally present. The authors suggested the interesting possibility that the material containing D-alanine and extractable with hot trichloroacetic acid may be an intermediate in the synthesis of cell wall protein.

TRANSAMINATION

For a survey and review of transamination, the reader is advised to consult the three informative general papers of Meister (2, 27, 28) that appeared in 1955. Reports continue to appear from time to time on the occurrence of specific transamination reactions without sufficient experimental evidence. Ideally, to show that a transamination reaction really occurs, one should obtain a quantitative balance between the reactants and the products. Minimum criteria for establishing such reactions should include quantitative determinations of the amounts of one of the expected products in an adequately controlled series of reaction mixtures. Information based only on visual examination of paper chromatograms may be misleading, and results obtained after long incubation periods, particularly with intact cells, should be viewed with the reservation that other reactions may have taken place.

Cell-free extracts of *Saccharomyces fragilis* catalyzed transaminations between α -ketoglutarate and a variety of L- and DL-amino acids [Bigger-Gehring (29)]. L-Aspartic acid and L-leucine were by far the most active of 22 amino acids tested. Transaminase activity was reported for intact cells of *Mycobacterium tuberculosis* by Hawkins & Pope (30). Glutamic acid was apparently formed from α -ketoglutarate and a series of L-amino acids. Aspartic acid- α -ketoglutarate transaminase was reported by Kanski *et al.* (31) to occur in intact cells and acetone powders of *Mycobacterium phlei*. Hicks (32) observed that carbon dioxide and alanine were produced on incubating aspartic acid and α -ketoglutarate with washed cells of *Clostridium perfringens* (*welchii*). Alanine was also produced from aspartic acid and either oxalacetate or pyruvate. The author assumed that the oxalacetate produced from aspartic acid was decarboxylated to pyruvate, and it in turn transaminated with some of the remaining aspartic acid (or glutamic acid) to form alanine. Cells and cell-free extracts of the Reiter spirochete catalyzed a series of transamination reactions as shown by Barban (33). Glutamic and aspartic acids and alanine were formed from their keto analogs, and glycine was formed from glyoxylic acid. Scher & Vogel (34) surveyed a number of microorganisms for the presence of ornithine δ -transaminase, which catalyzes the transfer of the δ -amino group of ornithine to α -ketoglutarate. The reaction occurred in extracts of *Neurospora sitophila*, *Torulopsis utilis*, bakers' yeast, *B. subtilis*, and *Bacillus pumilus*, but not in *E. coli*, *A. aerogenes*, *Erwinia carotovora*, *Serratia marcescens*, and *Proteus vulgaris*.

Transaminases for D-amino acids were found in *B. subtilis* and *Bacillus anthracis* [Thorne, Gomez & Housewright (35); Thorne & Molnar (36)]. Sonic extracts contained both L- and D-transaminases, but by ammonium sulfate fractionation preparations were obtained which were specific for D-amino acids. With purified preparations from *B. subtilis*, D-glutamic acid was synthesized by transamination between α -ketoglutarate and D-aspartic acid, D-alanine, D-methionine and D-serine. The L-isomers of these amino acids were inactive. Since alanine racemase was present even in purified transaminase preparations, both L- and D-alanine appeared to be active in

transaminating with α -ketoglutarate. However, the resulting glutamic acid was the D-isomer, indicating that only the D-isomer of alanine actually transaminated. D-Aspartic and D-glutamic acids transaminated with pyruvate to form alanine, but the L-isomers were inactive. A balance was obtained between the reactants and products in the reaction between D-glutamic acid and pyruvate, thus proving that transamination actually occurred.

Some of the D-transaminations catalyzed by extracts of *B. anthracis* were different from those demonstrated in *B. subtilis*. Several amino acids were tested and with the exceptions of glutamic acid and alanine, only the L-isomers transaminated with α -ketoglutarate, and only the D-isomers transaminated with pyruvate. As with preparations from *B. subtilis*, stability data and results of ammonium sulfate fractionation indicated that the *B. anthracis* enzymes catalyzing the D-transaminations were distinct from those active with L-amino acids. One of the most active reactions was that between D-phenylalanine and pyruvate forming alanine and phenylpyruvate. A balance was obtained showing that for each mole of alanine and phenylpyruvate formed, one mole of phenylalanine and one mole of pyruvate were used. *B. anthracis* also contained alanine racemase.

According to a recent report by Jordan (37) acetone-dried cells and cell-free extracts of *Rhizobium meliloti* catalyzed transaminations involving D-amino acids. Glutamic acid was synthesized in reaction mixtures containing α -ketoglutarate and D-aspartic acid or D-valine; alanine was produced from pyruvate and D-aspartic acid. Evidence was obtained for alanine racemase but racemases for aspartic and glutamic acids were not detected.

The occurrence of D-amino acid transaminases and alanine racemase suggests that alanine may be a connecting link between D- and L-amino acid metabolism in these organisms. The ability to carry out these reactions would alleviate the requirement for other racemases, e.g., L-glutamic acid could be converted to D-glutamic acid indirectly by transamination with pyruvate, racemization of the alanine, and transamination of D-alanine with α -ketoglutarate. Thus far, alanine racemase [Stewart & Halvorson (38); Wood & Gunsalus (39)] is the only amino acid racemase demonstrated in members of the *Bacillus* and *Rhizobium* genera, although glutamic acid racemase [Narrod & Wood (40); Ayengar & Roberts (41)], threonine racemase [Amos (42)], and methionine racemase [Kallio & Larson (43)] have been reported for *Lactobacillus arabinosus*, *E. coli* and a species of *Pseudomonas* respectively.

METABOLISM OF INDIVIDUAL AMINO ACIDS

Histidine.—The recent interesting work of Ames & Mitchell (44, 45) has contributed greatly to our knowledge of the mechanism of histidine synthesis in *Neurospora*. Previous work by Ames, Mitchell & Mitchell (46) had shown that histidine-requiring mutants of *Neurospora crassa* accumulated imidazoleglycerol, imidazoleacetol and L-histidinol in the growth medium. L-Histidinol had been isolated earlier by Vogel, Davis & Mingioli (47) from

an *E. coli* histidine mutant. None of these compounds replaced the histidine requirement of the *Neurospora* mutants and this suggested to the authors that these imidazole compounds might result from the breakdown of biosynthetic intermediates such as phosphate esters to which *Neurospora* mycelium is impermeable. Accordingly, upon fractionation of mycelium extracts imidazoleglycerol phosphate (IGP), imidazole-acetol phosphate (IAP), and L-histidinol phosphate were isolated. The structures of these compounds are shown in Fig. 1.

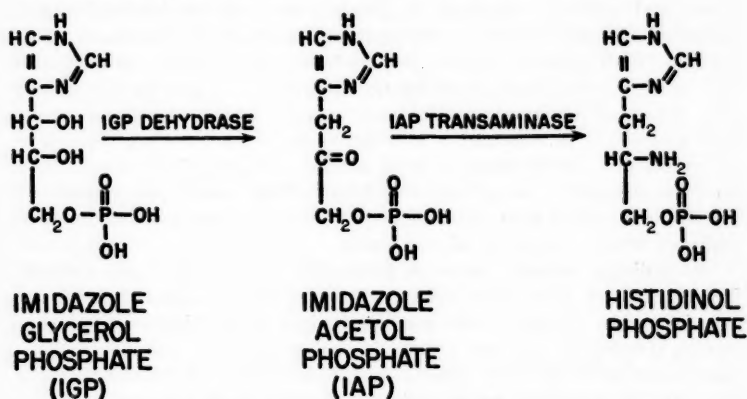


FIG. 1. Phosphate ester intermediates in histidine biosynthesis [Ames (45)].

Although none of these esters was active in replacing histidine for the mutants, very likely, as suggested by the author, because of the impermeability of *Neurospora* to phosphate esters, there is good enzymatic evidence that they are actually intermediates. Ames & Horecker [see Ames (45)] obtained evidence for an enzyme, IGP dehydrase, in cell-free extracts of *Neurospora* which converted IGP to IAP. A second enzyme, IAP transaminase, was found which catalyzed a transamination between IAP and glutamic acid to give histidinol phosphate and α -ketoglutarate.

Vogel, Davis & Mingioli (47) had found that L-histidinol was accumulated by one mutant of *E. coli* and metabolized by another. Adams (48, 49) discovered that cell-free preparations of yeast, *E. coli* and *Arthrobacter histidinolovorans* catalyzed the conversion of L-histidinol to L-histidine, and the presence or absence of the enzyme in histidine-requiring mutants correlated with the capacity of the mutants to substitute L-histidinol for their histidine requirement. L-Histidinal was also active as a substrate in the DPN-linked oxidizing system which converted L-histidinol to L-histidine, but it was inactive with preparations from *E. coli* mutants which were not able to carry out the complete oxidation. Evidence favored the suggestion that a single enzyme catalyzed the two successive oxidation steps from

histidinol to histidine. The following scheme for the biosynthesis of histidine is compatible with the data from both *Neurospora* and *E. coli*: IGP→IAP→histidinol phosphate→histidinol→histidinal→histidine.

Westley & Ceithaml (50) reported the isolation of 90 histidine-requiring mutants of *E. coli*. They concluded that the biosynthesis of histidine can occur by two pathways; the natural pathway involving L-histidinol as a terminal intermediate and the other pathway involving L-imidazole lactic acid.

Degradation of histidine by enzymes found in *Pseudomonas* extracts proceeds according to the following scheme [Tabor (51)]: L-histidine→urocanic acid→ α -formamidinoglutaric acid→formyl-L-glutamic acid→L-glutamic acid and formic acid. Magasanik & Bowser (52, 53) compared the pathways of histidine degradation in *Pseudomonas fluorescens* and *A. aerogenes*. Both organisms converted L-histidine to L- α -formamidinoglutaric acid. Extracts of *P. fluorescens* degraded this compound as outlined above, but extracts of *A. aerogenes* hydrolyzed it to formamide and glutamic acid. The fact that neither of the organisms was capable of hydrolyzing formamide is evidence that there are at least two different pathways of α -formamidinoglutaric acid degradation in the two organisms. The authors pointed out that this is the first instance of the appearance of formamide as a natural product.

Wachsman & Barker (54), working with *Clostridium tetanomorphum*, found an accumulation of formamide during fermentation of L-histidine or urocanic acid by resting cells. This observation suggested to the authors that urocanic acid is an intermediate in histidine fermentation by this organism and their results are consistent with the above scheme for histidine degradation in *A. aerogenes* although no evidence was obtained for the formation of α -formamidinoglutaric acid.

Another degradation pathway of histidine begins with the formation of histamine by the action of histidine decarboxylase and proceeds as follows: histamine→imidazole acetaldehyde→imidazole acetic acid→formyl-aspartic acid and NH_3 [Hayaishi (55); Hayaishi, Tabor & Hayaishi (56)].

Magasanik (57) made the interesting observation that the ratio of histidine assimilation and degradation depended on the concentration of histidine-degrading enzymes in the cell, and this in turn was controlled by the composition of the growth medium. The amount of L-histidine required for growth of *A. aerogenes* depended on the carbon source in the medium. Some compounds, e.g., glucose, permitted full growth with a supplement of 20 μg . of histidine per ml.; most compounds, e.g., *myo*-inositol, which could serve as sole carbon source for the organism, required 500 μg . of histidine per ml. These results were explained when it was learned that during growth on inositol the added histidine was degraded rapidly by adaptive enzymes whose synthesis it had induced, and that glucose was a powerful inhibitor of the synthesis of such enzymes. Acyl derivatives of histidine could supply histidine for cell protein synthesis but did not induce synthesis of the histi-

dine-degrading enzymes; thus acyl-histidines were more active than free histidine in promoting growth on the inositol medium.

Mathieson & Catcheside (58) studied the inhibitory effect of certain amino acids on the growth of three histidine-requiring mutants of *N. crassa*. The inhibitors were effective by preventing the uptake of histidine from the growth medium. Normally histidine was accumulated from the medium and stored in the mycelium from which it gradually disappeared. When amino acids which inhibited histidine uptake were introduced after histidine accumulation, they had no effect on the utilization of the stored histidine.

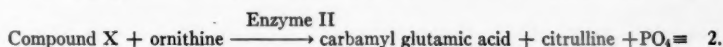
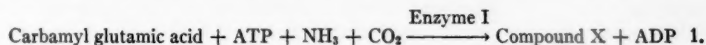
Miller (59) reported that peptides of histidine, e.g., glycyl-histidine, were essential for toxin production by *Clostridium tetani*. Free histidine supported growth but had no effect on toxin production. Acetyl-histidine could be substituted for the histidine peptides.

Valine, leucine and isoleucine.—In 1951 Tatum & Adelberg (60) proposed that in *Neurospora* isoleucine and valine shared a common precursor, a hypothetical 6-carbon dicarboxylic acid which was presumed to be formed by condensation of acetate with a 4-carbon unit related to threonine. From results of more recent experiments, however, Adelberg, Coughlin & Barratt (61) and Adelberg (62) have concluded that isoleucine and valine do not have a common origin in this organism. Isotopic tracer experiments confirmed the conclusion that exogenous L-threonine can supply four of the six carbon atoms of isoleucine and revealed that L-threonine is not a precursor of valine. The four carbons of threonine do not appear in isoleucine in continuous sequence however, and a pinacol type of rearrangement was postulated to give the carbon skeleton of isoleucine. Such a rearrangement had been proposed earlier by Strassman, Thomas & Weinhouse (63) to explain a similar finding in studies on biosynthesis of valine. In this and another more recent publication by these authors (64) evidence was obtained that in *T. utilis* the carbon chain of valine arises exclusively from pyruvate carbons. They suggested the following steps for the mechanism of valine synthesis: (a) decarboxylation of pyruvate to acetaldehyde; (b) condensation of acetaldehyde with pyruvate to form acetolactate; and (c) intramolecular migration of a methyl carbon from carbon 2 of the pyruvate moiety to carbon 1 of the acetaldehyde moiety of acetolactate to yield the keto analog of valine.

In isotopic competition experiments with *E. coli* Abelson (65) had shown that the addition of pyruvate, α -ketoisovalerate or L-valine resulted in lower specific activities of leucine synthesized from uniformly labeled glucose. He suggested that these substances are precursors of leucine and postulated that α -ketoisovalerate condenses with acetate to form α -ketoisocaproate, the keto analog of leucine. The results of Strassman and co-workers (66), obtained in isotopic tracer studies with *T. utilis* and supported by results Reiss & Bloch (67) obtained with *Saccharomyces cerevisiae*, are consistent with the findings of Abelson and point to a common origin of the isobutyryl moieties of leucine and valine. They proposed a scheme for the synthesis of leucine in which α -ketoisovalerate condenses with the methyl carbon of acetyl CoA

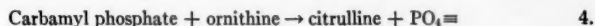
to yield α -hydroxy- α -isopropylsuccinate. This reaction would be analogous to the condensation of oxalacetate with acetate to yield citrate. By the same series of reactions undergone by citrate to form α -ketoglutarate, the postulated hydroxy acid would be converted to α -keto- γ -methylvalerate which could then form leucine by transamination.

Arginine, citrulline, and ornithine.—One of the year's most exciting and controversial topics was the composition of "Compound X." The conversion of ornithine to citrulline by enzymes in mammalian tissue had been shown to consist of two main steps [Grisolia & Cohen (68, 69); Grisolia (70)]:



Other acyl derivatives of glutamic acid, e.g., acetyl glutamic acid, could substitute for carbamyl glutamic acid.

The structure of Compound X had not been determined although these workers concluded that it contained one mole of each of acyl glutamic acid, CO_2 , NH_3 and phosphate. Recently Jones, Spector & Lipmann (71), using *S. faecalis*, showed that carbamyl phosphate was active as the precursor of the carbamyl group in citrulline. Synthetic carbamyl phosphate behaved the same as the compound formed enzymatically by incubating an equilibrium mixture of ammonium carbonate-carbamate with ATP and an extract of *S. faecalis*. They showed further that carbamyl phosphate would donate the carbamyl group to ornithine in a test system with rat liver mitochondria. These workers then formulated the synthesis of citrulline as follows:



Grisolia, Wallach & Grady (72) confirmed the findings of Jones *et al.* (71) when they showed that carbamyl phosphate and Compound X reacted equally well in reaction no. 4 above with partially purified preparations from calf liver or with extracts of Group D streptococci. From this and other characteristics of the two compounds, the authors concluded that Compound X and carbamyl phosphate were closely related. They maintained the two were not identical, however, on the basis of analytical data obtained on purified Compound X as well as on the basis of a molar relationship previously shown between carbamyl glutamic acid added to incubation mixtures and Compound X synthesized [Grisolia & Marshall (73)]. In their communication on "Biosynthetic and Structural Relationships of Compound X and Carbamyl Phosphate," Grisolia, Grady & Wallach (74) proposed that since the bacterial preparations, unlike mammalian preparations, do not require a glutamic acid derivative for the synthesis of reactive carbamyl groups, the bacterial enzyme might contain an active center with a structure similar to

acetyl glutamic acid and that carbamyl phosphate is released from the enzyme before further action.

In contrast to the view held by Grisolia *et al.* (72, 74), Marshall, Hall & Cohen (75) concluded that carbamyl phosphate is the intermediate carbamyl donor in enzymic citrulline synthesis by both mammalian liver and bacterial enzymes, i.e., it and Compound X are identical. Their conclusion was based on evidence which included the following: (a) They were unable to detect acetyl glutamic acid in a preparation which was presumably a purified sample of Compound X and which had been formed in the presence of acetyl glutamic acid; (b) new data showed there was no stoichiometric relation between acetyl or carbamyl glutamic acid added and Compound X formed; (c) compound X and carbamyl phosphate were converted to citrulline at the same rate by both a purified liver enzyme system and bacterial extracts; (d) compound X and carbamyl phosphate were converted to CO_2 , NH_3 and ATP at the same rate by extracts of *S. faecalis*. These authors also pointed out that in contrast to the bacterial system, the animal system requires catalytic amounts of a glutamic acid derivative for optimum synthesis of carbamyl phosphate.

Reichard & Hanshoff (76) reported that extracts of rat liver mitochondria catalyzed the formation of ureidosuccinic acid (carbamyl aspartic acid) from aspartic acid, NH_3 and CO_2 in the presence of acetyl glutamic acid, Mg^{++} and ATP. The synthesis was analogous to that of citrulline from ornithine, and the reaction proceeded in two steps. The first step involved the formation of Compound X, and the second step was the reaction between Compound X and aspartic acid to form ureidosuccinate. Extracts of *E. coli* also catalyzed the second reaction. After Jones *et al.* (71) reported on the activity of carbamyl phosphate in citrulline synthesis and also in the synthesis of carbamyl aspartic acid (ureidosuccinate) with extracts of *S. faecalis*, Reichard, Smith & Hanshoff (77) published the results of isotopic experiments dealing with the nature of Compound X. With rat liver mitochondria they obtained good evidence that (a) two radioactive carbamyl donors were formed in incubation mixtures containing citrulline-ureido- C^{14} , acetyl glutamic acid, and ATP; (b) the same two radioactive carbamyl donors were formed in reaction mixtures containing acetyl glutamic acid, NH_3 , $\text{NaHC}^{14}\text{O}_3$ and ATP; and (c) when Compound X was formed from radioactive carbamyl glutamic acid, NaHCO_3 , NH_3 , and ATP, two carbamyl donors were formed but only one was radioactive. In both (a) and (b) the formation of the two intermediates was dependent on the presence of acetyl glutamic acid. In each instance one of the isolated compounds behaved as synthetic carbamyl phosphate and the other appeared to contain carbamyl phosphate bound to acetyl glutamic acid (or carbamyl glutamic acid).

Since acetyl glutamic acid was necessary for the formation of carbamyl phosphate, the authors reasoned that Compound X (carbamyl phosphate

bound to acetyl glutamic acid) is probably the first intermediate formed in citrulline and ureidosuccinic acid syntheses and that carbamyl phosphate is formed from it. They suggested the reaction sequence shown in Fig. 2 for the mammalian system.

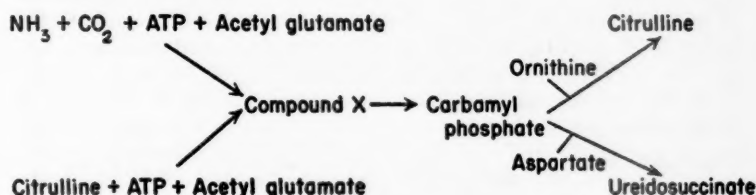


FIG. 2. Proposed sequence for reactions involving the carbamyl group [Reichard, Smith & Hanshoff (77)].

It appears that such a scheme is consistent with the findings of all four groups of workers. As Grisolia *et al.* (74) suggested, in the bacterial system Compound X or some similarly bound form of carbamyl phosphate may exist only in combination with the enzyme, i.e., the glutamic acid moiety may be an active center on the enzyme which releases carbamyl phosphate. In the mammalian system Compound X may occur as a free intermediate in the synthesis of carbamyl phosphate.

Matsushiro & Nakada (78) reported that an unidentified soil bacterium decomposed arginine to γ -guanidobutyric acid, CO_2 and NH_3 , and Thoai *et al.* (79) isolated a compound concluded to be guanidobutyramide from incubation mixtures of arginine and homogenized mycelium of *Streptomyces griseus*. Jackson & Pasioka (80) studied arginine degradation by *Micrococcus pyogenes* var. *aureus*. Intact cells degraded arginine to ornithine, NH_3 and CO_2 with no observable accumulation of citrulline, whereas acetone-dried cells degraded arginine to citrulline and NH_3 . Fleming & Foshay (81) observed that whole cells as well as sonic extracts of highly virulent strains of *Pasteurella tularensis* degraded citrulline to ornithine, CO_2 and NH_3 but strains of low or no virulence had no activity. The authors pointed out that this is the first metabolic difference thus far found between virulent and avirulent strains of this organism. None of the strains degraded ornithine.

Glutamic acid, glutamine and proline.—Cells and sonic extracts of *P. tularensis* oxidized glutamic acid as observed by Kann & Mills (82). The oxidation was dependent on the presence of phosphate, and with extracts, addition of DPN was necessary. Anaerobically, in the presence of triphenyltetrazolium chloride, glutamic acid was oxidized to α -ketoglutarate. Izaki, Takahashi & Sakaguchi (83) reported that several strains of bacteria metabolized the D-isomer of glutamic acid more rapidly than the L-isomer. When one of the strains was grown on a medium containing DL-glutamic

acid, the D-isomer was used completely but the L-isomer was recovered almost quantitatively. When 0.005M arsenite was added, α -ketoglutaric acid accumulated.

Two investigators reported on the pathway of glutamic acid fermentation by resting cells of *C. tetanomorphum*. Fry (84), studying the degradation of glutamic acid-1- C^{14} , found no C^{14} in the CO_2 produced, and all the C^{14} added was recovered in the fatty acids. Thus the CO_2 formed in the fermentation was not derived from the α -COOH group. The author suggested that α -ketoglutaric acid was not an intermediate since the usual metabolic route of this compound involves decarboxylation at the α -COOH group. Wachsmann & Barker (85) reported that the fermentation of L-glutamic acid by resting cells of *C. tetanomorphum* resulted in the formation of acetate, butyrate, hydrogen, CO_2 , and NH_3 . On the basis of a low yield of CO_2 and the origin of the carbon atoms in the products, the operation of a tricarboxylic acid cycle in the oxidative direction was excluded. Their finding that the CO_2 originated preferentially from carbon 5 of the glutamic acid is consistent with the report of Fry (84).

These investigations bring to mind the study of Tomlinson (86), who isolated glutamic acid from cells of *Clostridium kluyveri* grown in a synthetic medium with $C^{14}O_2$ or acetate-1- C^{14} as labeled substrates. With $C^{14}O_2$ 90 per cent of the C^{14} in the glutamic acid was found in the γ -COOH carbon, 7 per cent was in the α -COOH carbon, and the remainder was in the α -carbon. With acetate-1- C^{14} the C^{14} was found equally distributed between the α -COOH and the β -carbon. The conclusion was that the precursor of glutamic acid could not have been formed by Krebs cycle reactions.

Fry (87) observed that the ability to synthesize glutamine appears to be widespread in bacteria. Cell-free extracts of *M. pyogenes* var. *aureus* and a number of other bacteria catalyzed the formation of glutamyl-hydroxamic acid from glutamic acid and hydroxylamine in the presence of ATP and Mg^{++} . With extracts of *M. pyogenes* var. *aureus* glutamine was synthesized when hydroxylamine was replaced by ammonia. This direct test for glutamine synthesis was not applied to extracts of the other organisms, but it was assumed that since they could catalyze the synthesis of hydroxamic acid they could also form glutamine.

In further studies on the biosynthesis of γ -glutamyl peptides by transfer reactions, Williams, Litwin & Thorne (88) showed that with L-glutamine and D-glutamic acid together as substrates for an enzyme preparation from *B. subtilis*, the extent of the transamidation reaction resulting in synthesis of γ -glutamyl-glutamic acid exceeded that of the hydrolysis of L-glutamine under appropriate conditions. Prolonged incubation of L-glutamine, D-glutamic acid and enzyme resulted in the formation of glutamic acid peptides of increasing chain length up to at least six glutamic acid residues. The enzyme preparations also catalyzed a transpeptidation reaction in which γ -glutamyl radicals were transferred from the polypeptide produced by the organism to D-glutamic acid to form γ -glutamyl-glutamic acid.

Kogl, Emmelot & den Boer (89) used isotopically labeled D- and L-glutamic acids as substrates for glutamyl polypeptide synthesis by growing cultures of *B. subtilis*. They concluded that both isomers were incorporated into the polypeptide as D-glutamic acid, and postulated the presence of a glutamic acid racemase to explain their findings, i.e., the apparent conversion of L-glutamic acid to the D-isomer. Their attempts to demonstrate such a racemase were unsuccessful. However, their results could also be explained by taking into account the D-transaminases and alanine racemase of *B. subtilis*.

Waley (90) prepared synthetic poly-(γ -L-glutamyl)-L-glutamic acid and compared its properties with those of glutamyl polypeptides isolated from *B. anthracis* and *Bacillus licheniformis*. He concluded that the naturally occurring peptides were also γ -linked. This was in confirmation of the conclusion by Bruckner, Kovacs & Denes (91) that the polypeptides of *B. anthracis* and *B. subtilis* were made up predominantly of γ -glutamyl links and that few, if any, α -glutamyl links were present.

Abelson & Vogel (92) studied the biosynthesis of open chain amino acids and proline in *T. utilis* and *N. crassa*, using the isotopic competition technique. In regard to glutamic acid and proline, their results confirmed the following sequence: glutamic acid \rightarrow glutamic γ -semialdehyde \rightarrow Δ^1 -pyrroline-5-carboxylic acid \rightarrow proline. Such a sequence had been proposed earlier for *E. coli*, *N. crassa* and *T. utilis* [Vogel (93)]. In experiments of Strecker & Mela (94) washed cells of a mutant of *E. coli* accumulated Δ^1 -pyrroline-5-carboxylic acid, the intramolecular cyclization product of glutamic γ -semialdehyde, when glutamic acid was the substrate. An enzyme partially purified from *N. crassa* by Yura & Vogel (95) reduced pyrroline carboxylic acid to proline. The reaction depended on the presence of reduced pyridine nucleotide; both TPNH and DPNH were active. The reaction was assumed to proceed as shown in Fig. 3. The name, pyrroline-5-carboxylate reductase was suggested for the enzyme.

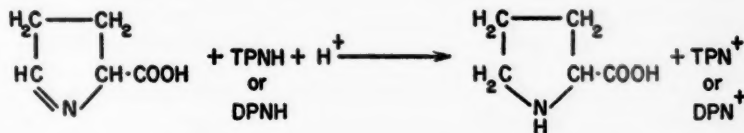


FIG. 3. Reduction of pyrroline carboxylic acid to proline [Yura & Vogel (95)].

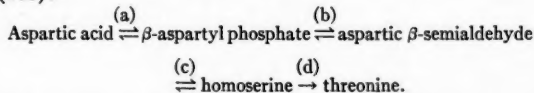
Serine and glycine.—Wang and his associates (96) studied the conversion of pyruvate-2- C^{14} to glycine and serine in brewers' yeast. The two amino acids were labeled very similarly in carbons 1 and 2, confirming their inter-conversion. A new cofactor necessary for the formation of glycine from serine by cell-free extracts of an unidentified species of *Clostridium* was obtained from boiled extracts of *Clostridium cylindrosporum* by Wright (97, 98). The

factor was designated as CoC. It would substitute for but was not identical with known folic acid derivatives. By fractionation of *C. cylindrosporum* extracts, five groups of pteridine derivatives with CoC activity were separated. They were believed to be polyglutamyl pteridine compounds which contained other amino acids as parts of the molecules.

A serine auxotroph of *E. coli*, studied by Meinhart & Simmonds (99), was able to use serine as a carbon source for the synthesis of glycine. However, the data indicated that another pathway of glycine synthesis occurred that did not involve serine as an obligatory intermediate. Campbell (100) studied the oxidative degradation of glycine by *Pseudomonas*. Cell-free extracts of an unidentified species degraded it to NH_3 , CO_2 and H_2O by way of glyoxylic and formic acids. The α -carbon was converted to CO_2 and the carboxyl carbon to formate.

Serratamic acid, which on acid hydrolysis yielded L-serine and an acid, tentatively identified as a hydroxyoctanoic acid, was produced by several strains of *Serratia* and was isolated in crystalline form from cell extracts by Cartwright (101). O-Carbamyl-D-serine was isolated from the culture medium of a new species of *Streptomyces*, *S. polychromagenus*, by Hagemann, Penasse & Teillon (102).

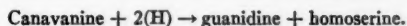
Aspartic acid, threonine and homoserine.—There has been further accumulation of evidence that L-aspartic acid is a precursor of threonine and experimental data support the following scheme as presented by Black & Wright (103):



The first reaction was catalyzed by an enzyme, β -aspartokinase, prepared from bakers' yeast by Black & Wright (104). β -L-Aspartyl phosphate and ADP were formed from L-aspartic acid and ATP. Step (b), the reversible reduction of β -aspartyl phosphate by TPNH to aspartic β -semialdehyde and inorganic phosphate, was catalyzed by another enzyme, aspartic β -semialdehyde dehydrogenase, isolated from yeast [Black & Wright (105)]. Step (c), the reversible reduction of aspartic β -semialdehyde to homoserine by DPNH or TPNH, was catalyzed by a third enzyme, homoserine dehydrogenase, also prepared from yeast by Black & Wright (106). All three enzymes were specific for the L-isomers.

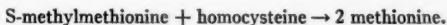
Nisman, *et al.* (107) [see also Black & Wright (103)] obtained evidence for reaction (d) in *E. coli*. Pyridoxal phosphate and ATP were required, and there was evidence that at least one intermediate occurred between homoserine and threonine. Watanabe & Shimura (108) reported the preparation of an enzyme from yeast that catalyzed the conversion of L-homoserine to L-threonine.

Resting cells of *S. faecalis* degraded canavanine according to the following equation [Kihara, Prescott & Snell (109)]:

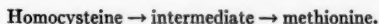


The authors suggested that the presence of an enzyme catalyzing this reaction in strains of *Neurospora* and *B. subtilis* may explain the equivalence of canavanine and homoserine in supporting growth of certain threonine-less mutants of these organisms.

Sulfur amino acids.—Shapiro (110) reported that a combination of methyl methionine sulfonium salt and homocysteine replaced methionine for growth of two methionine-requiring strains of *A. aerogenes*, and cell-free extracts catalyzed the synthesis of methionine from the two compounds. Schlenk & DePalma (111) found that methylmethionine sulfonium salt (S-methyl-methionine), as well as homocysteine, was less effective than methionine as a precursor of methylthioadenosine in yeast. A combination of the two compounds was highly active, however, and the results could be explained by the reaction:



Carlson & McRorie (112) obtained evidence for a new unidentified precursor of methionine in *N. crassa*. The presumed intermediate was found in extracts of a methionine-requiring mutant and was believed to be different from known intermediates in methionine synthesis. The authors suggested that it functions in the following sequence:



Kizer, Speck & Aurand (113, 114) observed that a combination of homocysteine and serine or phosphoserine reversed the retardation in growth of *Streptococcus lactis* in the absence of methionine. Phosphoserine was two to three times as effective as serine. The authors suggested that this latter observation supports the postulation of Arnstein (115) that in the β -oxidation of serine, the molecule is phosphorylated before cleavage and the phosphorylated carbon is transferred to homocysteine to form methionine.

An enzyme prepared from a species of *Pseudomonas* degraded methionine to NH_3 , CH_3SH and α -ketobutyric acid [Kallio & Larson (43)]. The enzyme was active only on the L-isomer and pyridoxal phosphate was the cofactor.

According to the interesting results reported by Gross & Tarver (116), when *Tetrahymena pyriformis* was grown in the presence of labeled ethionine, the label was incorporated into cell protein. When the labeled proteins were partially hydrolyzed, peptides containing labeled ethionine were found, indicating that the ethionine was an integral part of the protein.

An enzyme which catalyzed the synthesis of glutathione from glutamylcysteine and glycine was isolated from brewers' yeast by Snoke (117). The name, glutathione synthetase, was proposed for the enzyme. Its properties were almost identical with those of the glutathione-synthesizing enzyme previously isolated from pigeon liver by Snoke *et al.* (118). ATP and Mg ions were required for activity and potassium ions were stimulatory. Snoke &

Bloch (119) studied further the mechanism of action. The enzyme catalyzed an exchange of phosphate between ATP and ADP in the presence of Mg^{++} . The synthesis of glutathione was reversible, and the same enzyme catalyzed an exchange of the glycine moiety for free glycine or for hydroxylamine. Glutathione reductase was isolated and partially purified from a strain of *E. coli* by Asnis (120) and from bakers' yeast by Racker (121).

MISCELLANEOUS

Washed cells of *Chromobacterium violaceum* formed 5-hydroxytryptophan from L-tryptophan in experiments of Mitoma, Weissbach & Udenfriend (122).

In *E. coli*, as in *Neurospora*, the carboxyl group of anthranilic acid is removed during the formation of indole, and therefore in the synthesis of indole from anthranilic acid, two carbon atoms need to be added to form the pyrrole ring. Yanofsky (123) concluded from data obtained in isotope experiments with *E. coli* that the two carbon atoms were derived from carbons 1 and 2 of glucose by one pathway and from carbons 2 and 3 of glucose by a second pathway. Other experiments with extracts of *E. coli* had shown that the ribose derivative, 5-phosphoribosylpyrophosphate, participated in the conversion of anthranilic acid to indole [Yanofsky (124)], and the author suggested that the ribose derivative is the actual donor of the two carbon atoms.

Cell-free sonic extracts of *Brucella abortus* contained an enzyme which catalyzed the formation of pantothenic acid from potassium pantoate and β -alanine [Ginoza & Altenbern (125)]. The enzyme required ATP and was stimulated by Mg ions. An enzyme present in extracts of *Clostridium propionicum* catalyzed a reaction between acrylyl CoA and ammonia to form β -alanyl CoA [Stadtman (126)]. The author observed that the synthesis of the latter compound is the first demonstration of a biologically produced CoA derivative of an amino acid. The enzyme also catalyzed the corresponding reaction between acrylyl pantotheine and ammonia to form β -alanyl pantotheine.

Ikawa & O'Barr (127) isolated diaminopimelic acid from cell hydrolysates of *L. arabinosus*. It occurred to the extent of about 1 per cent of the dry weight of the cells. The formation of dipicolinic acid (pyridine-2,6-dicarboxylic acid) during sporulation of *Bacillus cereus* var. *mycoides* was studied by Perry & Foster (128). From results of experiments with C^{14} -labeled diaminopimelic acid, the authors concluded that this compound can be converted to dipicolinic acid. One proposed mechanism, i.e., ring closure of the carbon chain without prior rupture, would be analogous to that of the conversion of lysine to pipelicolic acid [Schweet, Holden & Lowy (129)].

In an investigation of the role of L-alanine in spore germination, Harrell & Halvorson (130) were unable to detect any metabolism of the amino acid during germination. An activation of spores occurred during exposure to L-alanine for 45 seconds. When alanine-1- C^{14} was used in the activation ex-

periments, only trace amounts of C^{14} were fixed in the spores. Incubation of adenosine with spores of *B. cereus* resulted in its cleavage to free adenine and ribose, whether or not other conditions, i.e., pH and temperature, permitted germination of the spores [Lawrence (131, 132)]. Spores of *Bacillus polymyxa*, which also required adenosine for germination, likewise cleaved it to adenine and ribose. However, spores of *Bacillus globigii*, which did not require adenosine for germination, and vegetative cells of *B. cereus* had no effect on adenosine.

The fate of C^{14} -labeled uracil in a pyrimidine-requiring strain of *E. coli* was investigated by Moore & Boylen (133). Uridylic and cytidylic acids and thymine isolated from the organism had the same C^{14} activity on a molar basis as the uracil supplied.

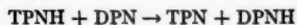
Five wild-type, as well as three purine-requiring strains of *E. coli* accumulated 4-amino-5-imidazolecarboxamide during growth [Slotnick & Sevag (134)]. Love & Gots (135) found a purine-requiring strain of *E. coli* which accumulated a new pentose arylamine. Cells of another mutant were able to convert the new amine to what was apparently a pentose derivative of 4-amino-5-imidazolecarboxamide. The authors suggested that the new unidentified amine might normally function as a precursor of a pentose derivative of 4-amino-5-imidazolecarboxamide and consequently of purine derivatives.

Growing cells and washed cell suspensions of *Pseudomonas aeruginosa* degraded uric acid to oxalic acid, NH_3 and CO_2 , as reported by Franke & Hahn (136), who identified allantoin, allantoic acid, glyoxylic acid, and urea as intermediates. Campbell (137) obtained cell-free extracts of an unidentified species of *Pseudomonas* which degraded uric acid to urea, CO_2 , and water. Apparently the pathways of degradation by the two organisms were identical up to glyoxylic acid and urea. *P. aeruginosa* oxidized glyoxylic acid to oxalic acid and hydrolyzed urea to NH_3 and CO_2 , while the other species oxidized glyoxylic acid to CO_2 and water via formic acid but did not hydrolyze urea.

Guanase was isolated from culture filtrates of *Clostridium acidurici* by Rakosky, Zimmerman & Beck (138). It deaminated guanine to xanthine, and 1-methyl guanine and 8-azaguanine were likewise converted to their respective xanthine derivatives.

In experiments with disrupted staphylococcal cells Gale & Folkes (139) showed that incorporation of specific amino-acids into protein could be activated by specific combinations of nucleotides, i.e., for the incorporation of a particular amino acid, a specific combination of nucleotides could replace intact ribonucleic acid. They suggested that the effect of ribonucleic acid lies in the presence of such combinations within the polynucleotide structure.

Results of experiments done by San Pietro, Kaplan & Colowick (140) indicate that the reaction,



which is catalyzed by the pyridine nucleotide transhydrogenase from *P. fluorescens* [Kaplan, Colowick, Zatman & Ciotti (141)], involves a direct hydrogen transfer rather than electron transfer. The stereo-specificity of this enzyme was shown to be opposite to that of yeast alcohol dehydrogenase.

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PROBLEMS OF INCUBATION IN PLANT DISEASES¹

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In the course of an infectious disease, we may roughly distinguish four main periods: (a) the period of infection during which the parasite enters into the host (1), (b) the period of incubation which follows infection and ends with the appearance of the first disease symptoms, (c) the period of obvious disease, and (d) the period of recovery.

The present article deals with processes occurring during incubation. In this period, the parasite tries to grow, to proliferate and to spread in the host tissues. Furthermore, it may exert characteristic pathogenic actions, and the host plant undergoes the first pathological alterations. These initial processes consist in biochemical and physiological reactions inside of tissues and cell protoplasts; they are in general inconspicuous and difficult to follow. Only in a later stage (i.e. at the end of incubation) do morphological alterations become visible on the plant. These visible signs are often unspecific and cannot give much information about the mechanisms of pathogenic action. External symptoms on the plant are the expression of profound alterations or even of the death of cells and tissues, and the really important and specific reactions may already be over. If we want to penetrate into the fundamentals of host-parasite relations, we have to concentrate on the early processes which decide the fates of host and parasite.

The complex nature of these processes becomes evident when we compare the typical features of various plant diseases: the differences in length of incubation, in the parasites' extension in the host, in the specific attack of tissues and organs, etc. In many diseases, the sequence of visible events is rather well known; about the reasons and factors which determine this sequence of events, only scattered information is available. Therefore, this article cannot be a complete survey of well known subjects; we shall try instead to point out some problems which are under investigation but still need much better understanding. This, of course, implies a rather subjective selection of literature. Three main aspects will be discussed in this review: (a) the factors enabling a parasite to grow and to spread in the interior of the host at the host's expense, (b) the actions of the parasite which cause a disease of the host, and (c) the first pathological reactions of the host.

Many related problems of plant diseases and diseases in general have been treated in recent textbooks and reviews (1 to 8) and in an extensive symposium (9).

GROWTH AND EXTENSION OF THE PARASITE IN THE HOST

In this chapter, the parasite is regarded not as an organism causing disease but as an organism struggling for life under the conditions given by the

¹ The survey of literature pertaining to this review was completed in January, 1956.

host plant. Whether the parasite is able to survive, to nourish itself, to proliferate and to spread in the host's interior depends on a series of factors on the part of the host and on the part of the parasite. The complex of properties enabling an organism to live as a parasite is called aggressiveness (2, 3); on the part of the host, various factors determining susceptibility or resistance are involved in these processes.

THE NUTRIENTS IN THE HOST PLANT

For the invading parasite, the host plant represents first of all a substrate to feed upon. The presence of suitable nutrients and the ability of the parasite to utilize them under the given conditions are an important prerequisite for its growth and proliferation. The question of the nutritional requirements of plant parasites has often been raised, in particular in connection with obligate parasites such as rusts or mildews, which today cannot yet be grown *in vitro* with ordinary laboratory techniques. To what extent are nutritional requirements involved in the specificity of these fungi for certain host plants, and to what extent do these parasites depend on specific intermediate substances of the host metabolism (10)?

The relationships between nutritional states of hosts and the development of obligate parasites have been reviewed by Allen (5). On one point, a rather direct connection has been established: in a susceptible host plant, an adequate supply of carbohydrates is necessary for the development of obligate parasites. For example, barley plants grown under extreme short day conditions (two hr. of light per day) form less assimilates than normal plants, and the parasite, *Erysiphe graminis hordei*, shows only poor growth. Application of glucose to these etiolated leaves increases the development of the parasite (11). In plants grown under long day conditions (17 hr. of light per day), growth of the parasite is likewise poor; in this case, lack of carbohydrates is not the limiting factor, and the provision of glucose remains without influence. In this last example as well as in many others we can only ascribe resistance or susceptibility to the general metabolic state of the host (which of course is influenced by external factors such as nutrition, temperature, light, etc.) and we do not yet know the exact nature of the internal factors involved. In several cases of facultative parasites, for instance, a high sugar content goes parallel not with an increased, but with a reduced development of the parasite (12, 13, 14). In these examples, the situation seems less clear than in the case of the obligate parasites mentioned before.

Tracer studies with rusts and mildews have shown that phosphates, sugars, and other products of carbon dioxide assimilation accumulate at the site of infection (15, 16). Within or on the haustoria of *E. graminis*, relatively high concentrations of acid phosphatases are present (17). These observations indicate an increased metabolic activity in the infected area; details remain open for future studies. Continued research on the *in vitro* culture of rusts (18, 19) and on the significance of phosphorylated metabolic intermediates as discussed by Cutter (10) should help to answer the question as to what really happens in the infected host.

For various plant pathogens a correlation between aggressiveness and nutritional requirements has been demonstrated with the aid of biochemical mutants. *Erwinia (Bacterium) aroideae*, when grown on the cut surface of cucumbers, potatoes, radishes, etc. causes brown discoloration and rot of the invaded tissues. Several mutant strains deficient in synthesis of a particular amino acid have been obtained which are devoid of aggressiveness for some of these hosts (20, 21). All mutants remain aggressive for cucumbers. Mutants requiring arginine, threonine, or cysteine lost their aggressiveness for radishes, whereas mutants requiring methionine, isoleucine and valine together, leucine, or histidine kept it. Addition of the required amino acid to the site of infection with a non-aggressive strain led to the typical brown discoloration and rot. Addition of a host tissue block to an agar plate permitted growth *in vitro* of an aggressive but not of a nonaggressive strain. In the cases where aggressiveness is lost, the required amino acid apparently is not available at the cut surface in a quantity big enough for the development of the parasite; the addition of tissue homogenates to agar plates permitted growth of the parasites *in vitro* in all combinations. Similar results have been obtained by Keitt & Boone (22) with mutants of the apple scab fungus, *Venturia inaequalis*. Mutants deficient for nicotinic acid, biotin, inositol, or pantothenic acid retained aggressiveness and pathogenicity. Mutants deficient for choline, riboflavin, purines, pyrimidines, histidine, methionine, arginine, or proline caused only yellowish flecks or no symptoms at all. Here again it appears that the substances required are not present in quantities sufficient for full development of the parasite. Similar correlations between a nutrient deficiency (especially for purines) and a loss of aggressiveness have been reported for several bacteria pathogenic for man (23, 24, 25). A race of *Phytophthora infestans* pathogenic to various highly resistant potato hybrids grows on several amino acids which a less pathogenic race is not able to utilize (26); the significance of this difference has not yet been studied in detail.

ENZYMES OF PARASITES DURING INCUBATION

Growth, nutrition, and extension of a parasite depend to a great extent on its enzymatic properties. The question must be raised, therefore, to what extent specific features of host specialization, tissue specialization, etc. depend on the formation of particular enzymes.

Enzyme formation and adaptation.—Despite the tremendous work which has been done on the mechanisms of enzyme formation (cf. 27 to 30) many points are still controversial. Much attention has been given to the two groups of enzymes: the constitutive, which are regularly formed by the cell, and the adaptive, which are formed only in the presence of the specific substrate. It seems, however, that the formation of both enzyme types follows the same rules and that the differences are of a quantitative nature. In the case of constitutive enzymes, a considerable amount of enzyme is present at any time, perhaps under the constant induction of normal cell constituents (31). In the instance of adaptive enzymes, an inductor (which

is usually but not necessarily the specific substrate) stimulates the formation of the enzyme from a precursor. Even in the absence of the inductor the enzyme is formed in small quantities. Based on this concept, the term "induced enzyme formation" is preferred to "adaptive enzyme formation" (27). According to Dean & Hinshelwood (32), "the adaptive changes normally studied are quantitative developments of latent properties, and do not represent the sudden appearance of new qualities."

Most studies on enzyme formation by plant parasites concern pectic enzymes. Pectin-splitting enzymes are formed as constitutive or inducible enzymes by many fungi or bacteria (33 to 38). Pectinmethylesterase is formed as an inducible enzyme by *Botrytis cinerea*, *Aspergillus niger* and *Penicillium chrysogenum*; polygalacturonase is formed as a constitutive enzyme by *B. cinerea* and *A. niger* but as an inducible enzyme by *P. chrysogenum* (33, 34). That an enzyme may be constitutive in one strain and inducible in another strain of the same species has been shown with mutants of *Escherichia coli* (31). The reasons for these differences are not yet clear. Enzyme formation is influenced not only by the specific substrate or structurally related substances but also by other environmental factors. The amount of polygalacturonase produced by *B. cinerea* and *Pythium de Baryanum* *in vitro* depends to a great extent on the ratio of carbohydrates and nitrogenous compounds in the nutrient solution and on other nutritional factors (36, 37). We may safely assume that enzyme production by the parasite during incubation may similarly be influenced by various host constituents. The implications of such effects for the host range of various parasites and other problems remain to be elucidated.

Rôle of enzymes in the extension of parasites.—In human pathology, considerable attention has been given to factors which increase the permeability of host tissues to parasites and their metabolic products. Among the best known are the hyaluronidases. This enzyme complex disintegrates hyaluronic acid which forms intercellular cementing material of tissues. The problems involved are complicate; there are other factors which may also increase the invasiveness of parasites and the permeation of their toxins [lecithinases, collagenases, histamin-like substances, etc. (30, 39 to 42)].

Similar processes must certainly go on in plant diseases even if little conclusive evidence of this is available at the present time. They may play a rôle in the development of fungi which at the beginning of incubation live in the intercellular spaces and only later shift to an intracellular habit. *Cladosporium cucumerinum* grows first in the intercellular spaces of susceptible cucumber tissues without doing much damage to the host cells. Three to five days after infection the fungus penetrates the host cells where it soon causes serious pathological reactions (43). The production of some enzymatic "spreading factor" during the early incubation period might be involved in this sequence of events.

The question of enzymes enabling certain parasites to spread in the host plant has been discussed in connection with tomato wilt caused by *Fusarium lycopersici*. Pathogenic and nonpathogenic strains of this parasite are known, all of which infect tomato roots from the soil. The nonpathogenic strains

remain localized in the roots; they continue to live in the roots and may be reisolated from them but do not penetrate the stem. Pathogenic strains grow upwards in the xylem of stems and petioles; they cause a browning of vessels, and finally necrotic leaf spots and the death of the plant. The question of what factor enables the pathogenic strains to extend into the stem has been raised. White (44) suggested the formation of enzymes, and during recent years various enzymes have been examined in connection with *Fusarium* wilt of tomato without leading to definite conclusions. Pectin-methylesterase may cause browning of vessels in tomato cuttings but it is formed by non-pathogenic as well as pathogenic strains and apparently cannot explain differences in pathogenicity (45).

Non-enzymatic substances may have similar effects on the host plants. In cabbage yellows, radish wilt, and cotton wilt *Fusaria*, Winstead & Walker (46) describe thermostable, dialyzable metabolites which intoxicate disease-susceptible host plants only and may contribute to the establishment of the parasite in these hosts. The exact chemical nature of these metabolites is not yet known. Among the substances which might facilitate spreading of parasites and toxins, trimethylamine has been mentioned (47); this penetrates and destroys plasmatic layers easily and is known to be produced by fungi.

Certain plant parasites apparently lack the capacity to attack native cellulose in the plant host, and proliferate in the middle lamellas of their host tissues. Unfortunately, the enzymes involved in cellulose decomposition are not yet exactly known. Reese, Siu & Levinson (48) distinguish a Z_1 enzyme which splits native cellulose into linear anhydroglucose chains, and a Z_x enzyme which hydrolyzes the 1,4- β -glucosidic linkages of these chains to soluble molecules of a low molecular weight. Other authors (49, 50) reject this concept and assume that there exists only one enzyme which under certain conditions fails to split native cellulose. Ammann (51) has shown that water soluble methyl cellulose is attacked by culture filtrates of numerous fungi and bacteria, even those white rot fungi which attack especially the lignin of the wood (*Polystictus versicolor* and *Schizophyllum commune*), and also *Erwinia carotovora* (*Bacterium carotovorum*) which grows in the middle lamellas of potatoes. The enzyme involved (corresponding to the Z_x enzyme of Reese *et al.*) is constitutive for these organisms. The question of native cellulose decomposition remains open; work on this line as well as with lignin decomposing enzymes implies considerable methodical difficulties.

Summarizing this section we might say that we are inclined to ascribe various features of plant diseases to the action of enzymes but that conclusive proofs are still rare. We need more information about the genetically fixed enzymatic-properties of the parasites, about the formation and activity of these enzymes under the specific conditions in the host, about the specificity in enzyme induction and many other questions which enzymologists cannot answer at the present time.

PHYSICO-CHEMICAL CONDITIONS IN THE HOST

In human pathology, acidity and oxidation-reduction potentials have a great influence on proliferation and organ localization of bacteria. Some

bacteria are rather strictly aerobic or anaerobic, others are less definite in their requirements. Oxidation-reduction potentials are influenced not only by the oxygen supply but also by the presence of reducing substances such as cysteine or ascorbic acid; in the presence of cysteine, anaerobic bacteria may be grown aerobically (52). Few recent studies deal with plant pathological problems of this kind. As far as is known, the growth of plant parasitic fungi and bacteria is not much affected by pH and rH changes over a wide range (2, 3); on the other hand, the formation, penetration and action of enzymes and toxins are definitely influenced by such changes. *In vitro* experiments with the wilt toxin fusaric acid might illustrate this. Fusaric acid inhibits the respiration of yeast cells (53). The toxic effect itself is almost independent of pH, but penetration of the substance into the cells depends to a great extent on the pH of the surroundings. Corresponding to the general experience that undissociated molecules penetrate protoplasm much better than dissociated ones (54), the quantity of toxin absorbed by the yeast cells at pH 4.5 is four times the quantity absorbed at pH 7.8. The final inhibition of respiration, therefore, depends on the concentration as well as on the dissociation of the toxin. Examples of pH influence on cellulose splitting enzymes are given by Siu (55).

Permeability problems have already been touched upon in a previous section. There can be no doubt about the general importance of permeability in normal and diseased life processes of all organisms (56, 57). Complicated metabolic and enzymatic processes going on at the surface of protoplasts regulate the uptake and release of substances (58, 59). Permeability increase in infected plants will be discussed later; decrease may be involved in defense reactions of the host plant. In certain resistant wheat races, penetrating hyphae of *Puccinia graminis* cause a local decrease of permeability in the adjacent cells. Metabolic exchange reactions and food uptake of the parasite are more and more reduced and lead to the death of the fungus (60).

DEFENSE MECHANISMS OF THE HOST

Growth and spread of a parasite in the host may be affected by mechanical barriers, toxic substances and other factors of resistance, and by the defense reactions of the host (2, 3). Only a few aspects will be mentioned here.

Toxic substances as factors of resistance.—Walker & Stahmann (8) have discussed the chemical nature of disease resistance and the difficulties of interpretation encountered in these problems. The significance of a substance like a phenol, tannin, or alkaloid in plant resistance depends not only on its presence in resistant host species and on its fungitoxicity but also on its qualitative and quantitative distribution in the host tissues, on solubility (61), or fixation to other cell constituents, and on the enzymatic properties of the parasite. The following example might illustrate the situation: tomatine, a glycosidic alkaloid in tomato plants, inhibits in high concentrations the growth of certain fungi *in vitro*; however, the tomato wilt fungus *Fusarium lycopersici* is even stimulated in growth and probably utilizes the sugars contained in the molecule (62). This observation together with the low con-

centrations of tomatine in roots and stems of resistant and susceptible tomato plants does not favour the idea of tomatine as a factor of resistance in tomato wilt (62, 63, 64).

Defense reactions of the host.—A host plant represents not only a substrate of a certain chemical composition but a living organism, and it is therefore capable of answering the parasite's actions in different ways. The intensity of these reactions varies to a great extent. The mycelium of *Gymnosporangium sabiniae* may live for years in the same bark tissues of *Juniperus* species without doing heavy damage to it and without causing serious reactions. The hyphae grow between the host cells and form appressoria to phloem and other cells (65). Systemic smut fungi may grow and spread in a similar way without inducing severe host reactions. *Rhizoctonia* fungi in orchid bulbs and *Rhizobium leguminosarum* in the roots of leguminous plants are restricted by host reactions (2, 3, 66, 67) to the external parts of the host but may live there for rather long periods. In many of these cases, incubation is very long and without a sharp limit.

In other diseases, early and intense host reactions disturb this quiet development and determine the sequence of events. They may lead to necroses and death of host cells and parasite (as in the wart disease of potatoes), or to a demarcation and isolation of the site of infection (as in the shot-hole disease of stone-fruit trees). The different types of defense reactions have been extensively described by Gäumann (2, 3, 66, 67); the biochemistry of the inducing substances and of the host reactions still remains to be elucidated. In the case of the shot-hole disease of stone-fruit trees, fungal metabolic products as well as decomposition products of destroyed host cells (necrohormones) spread from the infection site into the surrounding tissues. At a distance of some cell layers a small zone of the leaf tissues becomes meristematic again; it becomes lignified and suberized and finally surrounds the infection focus as a firm demarcation zone. The attacked part of the leaf is isolated and the fungus does not spread further into the surrounding tissues (68). By injection of fungal toxins, inorganic or organic poisons, or by heavy lesions of the leaf, the reaction may be induced through decomposition products of destroyed host cells alone. In the bitter rot disease of cherries (caused by *Glomerella rufomaculans*) similar demarcations are induced but develop too slowly compared to the mycelial growth; the fungus breaks the demarcation tissues again and again and spreads over the whole leaf (69).

In several diseases, a close connection between resistance and the metabolic activity of the host has been established (70, 71, 72). In tomato races resistant to Fusarium wilt, resistance is broken by treatment with 2,4-dinitrophenol or other respiratory inhibitors (73). On the other hand, application of 2,4-dichlorophenoxyacetic acid makes susceptible tomato plants resistant (74). The concentration of the growth factor required for this effect is far below that which inhibits growth of the fungus *in vitro*; the effect of the treatment increases as the interval between drug application and inoculation with fungus is prolonged, and apparently depends upon metabolic

alterations. The formation of antifungal substances as a reaction to metabolic products of a parasite has been demonstrated with orchid bulbs (75). Some orchids carry a mycorrhizal fungus in their roots but not in their bulbs. If such a fungus grows on an agar surface towards a living bulb, the mycelium does not reach the bulb but stops at a certain distance from it. A bulb killed at low temperatures is covered by the mycelium in a short time. It may be assumed that metabolic products moving ahead of the growing mycelium induce in the bulb the formation of a substance which in turn diffuses from the bulb and at a certain concentration hinders the mycelial growth of the fungus. The chemical nature of this induced antifungal factor is under investigation. An apparently similar mechanism has been described recently by Kuć, Ullstrup & Quackenbush (76). Potato peels contain a substance inhibitory to the growth of *F. lycopersici* and other fungi nonaggressive for potatoes. Potato pulp tissue does not contain such a substance but may form it after inoculation with one of these fungi. Consequently, inoculation does not succeed. The substances involved are nonspecific insofar as a substance produced as a response to one fungus inhibits growth of the others too. In potatoes resistant to *P. infestans*, an increase in polyphenol substances following inoculation has been found (77). It makes an interesting task for future studies to elucidate the biochemical nature of these metabolic processes and their significance for resistance.

THE PATHOGENIC SECRETIONS OF THE PARASITE

During their growth in the host, the parasites secrete various metabolic products which accumulate in the surrounding tissues or are carried in the transpiration stream into distant parts of the plant. The presence of these parasite-borne substances in the host may be demonstrated with the aid of radioactive tracers (78, 79, 80). Some of these substances are harmless, others cause pathological reactions in the host. In the following survey we shall roughly divide these pathogenic secretions into enzymes, toxins, and growth factors.

ENZYMES

As in infections of animals, some of the toxic substances secreted by plant parasites certainly are enzymatic in nature. The action of cellulose or lignin splitting enzymes may be followed in the destruction of wood by many fungi; detailed information, however, is rare in this field. Despite a number of detailed studies, the rôle of enzymes in tomato wilt caused by *F. lycopersici* is not yet quite clear (7). Pectic enzymes and β -glucosidases have been discussed as factors causing vessel browning and wilt (45, 81 to 88). These latter enzymes are supposed to liberate phenols from glucosides; the phenols in turn may be oxidized to melanin-like pigments which cause vessel browning. Vasinifuscarin, which also causes vessel browning, is probably an enzyme too but has not yet been identified (89).

TOXINS

In this group we consider those toxic metabolic products of parasites which do not typically act as enzymes or growth factors. As far as is known

they interfere with host metabolism by various biochemical mechanisms and finally cause leaf necroses, shrinking and collapse of tissues, stem lesions, etc. There is of course no sharp distinction between these three groups of toxic substances, and future studies may change this picture considerably. We shall discuss the formation of toxins *in vitro* and *in vivo* and the chemical nature of toxins; the biochemical mechanisms of action will be described later.

Toxin formation in vitro and in vivo.—The direct isolation of a toxin from the host plant is extremely difficult. For this reason, toxins are in most cases extracted from pure cultures of the causative organism. This, of course, implies certain difficulties. Studies *in vitro* give indications of the general biochemical capacities of this organism, but they do not permit direct conclusions concerning the effective activities of the organism under other conditions. On the usual Richard medium, the nonpathogenic strain 257 of *F. lycopersici* produces more toxic substances (measured by Wilt and spore germination tests) than the pathogenic strain R-5-6 (62); if the same solution with only half of glucose and ammonium nitrate is used, toxin production is higher in strain R-5-6 (90). Despite these restrictions, *in vitro* studies are necessary. With the knowledge of the chemical composition of a toxin, methods of determination may be developed which permit the demonstration of the presence of a toxin in the small quantities to be expected in an infected plant. Furthermore, toxin molecules may undergo rapid changes in the plant (90); in some cases it may be possible to isolate not the toxin itself but a derivative of it. Toxin formation *in vitro* may give even more information about its rôle *in vivo*. In several cases it has been shown that highest amounts of toxins or enzymes are formed during the early period of rapid mycelial growth (33, 62, 91). This holds also for lycomarasmin and fusaric acid which may be detected chromatographically after six or seven days of culture (90, 92). These results indicate the possibility of an action of such toxins in early phases of disease and may also contribute to an explanation of the rapid progress of some wilt diseases in early summer [e.g. in apricot trees attacked by *Valsa* fungi (2, 3, 62)]. With rising temperature the fungus grows better and better; more and more toxins are formed and cause a heavy and sudden intoxication of the tree.

Very little information is available about toxin formation in infected plants. The presence of fusaric acid in cotton plants inoculated with *Fusarium vasinfectum* has been shown with chromatographic techniques (93). In tomato plants inoculated with *F. lycopersici*, fusaric acid (or a closely related derivative) has been identified by the isotope dilution technique (94). Fusaric acid may therefore be regarded as a vivotoxin in the sense of Dimond & Waggoner (95). Fluorescence of infected cotton plants also makes the presence of toxins probable (96). The formation of other toxins *in vivo* will certainly be demonstrated in future studies.

The chemical nature of toxins.—The toxins known from bacteria pathogenic for man and animals are generally protein substances of high molecular weight (41, 97). The toxins isolated so far from plant parasites are comparatively small molecules belonging to different chemical groups (3, 98, 99). Among the chemical groups where toxins have been found are amino

acids (wildfire toxin), peptides (lycomarasin, enniatin), lactones (clavacin), and pyridine derivatives (fusaric acid). Some of the better known toxins shall be described in more detail in a later section. They represent only a small selection, and future studies will increase the list considerably. However, there may be an explanation for the prevalence of rather small molecules insofar as the cellulosic cell walls exert a filter effect and hinder the penetration of large molecules into the cell.

GROWTH FACTORS

In a great number of diseased tissues auxin concentration is known to increase; in a few cases only auxin production by the parasite has been shown. In the genus *Nectria* a correlation between auxin formation and pathogenic action has been established (100): *Nectria galligena*, causing tumors on apple tree branches, produces indoleacetic acid in culture, whereas *Nectria cinnabarina*, which causes no swellings, apparently does not. From cultures of *Gibberella fujikuroi*, the cause of bakanae disease in rice, gibberellin A has been isolated [$C_{22}H_{36}O_7$, chemical structure still unknown (101, 102)]; the substance has no auxin effects in a strict sense but when applied to rice seedlings causes an elongation of the stems and inhibition of root growth. Much work remains to be done in this field.

On the whole, we should not imagine the pathogenic actions of a parasite in too simplified a way. A single disease is often caused by a number of different factors which only in combination lead to the characteristic disease aspect. Toxic actions of the parasite only have been mentioned so far. Other factors such as interruption of the water flow in the vessels by fungal mycelium, tyloses, polymers secreted by the parasite etc., have been reviewed by Dimond (7). We cannot yet give complete pictures of the mechanisms operating in a diseased plant; analysis of single factors only seems feasible at the present time.

PATHOLOGICAL ALTERATIONS IN THE HOST

In many cases, pathological alterations of the host are initiated as biochemical reactions between host constituents and toxic metabolic products of the parasite. These toxic substances may spread in the host tissues or they may act in the immediate range of the parasite only where they are even more difficult to follow. In many diseases where specific mechanisms have not yet been shown, we have to assume an exchange of biologically active substances between parasite and host protoplasm. These basic biochemical processes lead to functional and also morphological alterations in the cells. These alterations become more and more pronounced; finally, the first external symptoms appear and the period of incubation comes to its end. The earlier the events in this reaction chain the more difficult they are to observe. It is not surprising that only scattered data are available at the present time. Some studies have been carried out with toxins of wilt diseases. Even if many of these investigations have been done *in vitro*, they will finally contribute to a better knowledge of the mode of action of the parasites.

BIOCHEMICAL REACTIONS

There are only a few examples wherein parts of the biochemical reactions of toxins have been elucidated. Clavacin, a lactone produced by *Penicillium* and *Aspergillus* species, is able to block about fifty per cent of the thiol-groups in tomato cuttings (103). Antibacterial action on the basis of combination with essential —SH groups is known also for arsenicals and other substances (104). Whether clavacin acts on thiol-groups in enzyme systems or on oxidation-reduction potentials cannot yet be decided. A second example is known from the wildfire bacterium *Pseudomonas tabaci*. This bacterium causes localized yellow halos on tobacco leaves which are free of bacteria and caused by a toxin diffusing from the infection center. The toxin has been identified as an α -amino acid and a structural analogue of methionine (105, 106), preventing normal utilization of this essential amino acid. The toxin therefore acts as an antimetabolite (107, 108). The toxic effect may be reversed by addition of methionine at a certain ratio. Methionine sulfoximine, another methionine analogue, causes similar intoxications.

Toxins may also act by formation of chelate complexes with metals (109). When lycomarasmin (a dipeptide produced by *F. lycopersici*) is applied to tomato cuttings, it forms water soluble iron complexes in the stem. The complexes are carried into the leaves where part of the iron is liberated again. Lycomarasmin, therefore, causes iron deficiency in the stem and iron excess in the leaves. Application of the equimolar lycomarasmin-iron-complex causes a heavier intoxication because additional iron is introduced into the plant; application of the stable equimolar copper-lycomarasmin-complex causes much less intoxication because most lycomarasmin molecules are blocked by the copper (110, 111). The nature of these iron effects is not yet known. Fusaric acid (5-n-butyl-picolinic acid produced by *F. lycopersici* and other fungi) forms water insoluble metal complexes; their biological rôle has not yet been definitely established. Lycomarasmin too may still have other effects besides complex formation. These examples may indicate some directions of research; in these and other cases, future studies have to show which cell sites, metabolic steps, enzyme systems, etc. are affected by the toxins. That toxins themselves may undergo chemical changes in the host plant (decarboxylation, etc.) has been shown recently with radioactive fusaric acid (90).

ALTERATIONS OF CELL FUNCTIONS

Permeability.—Among the cell functions disturbed in the course of an infectious disease, those involving permeability have received particular attention. Permeability of protoplasm is regulated by complicated processes closely connected with enzyme action at the cell surface (58), respiration, and general cell metabolism (56, 59), and it is still difficult to understand. Permeability increase during incubation is a wide-spread phenomenon in human pathology (57, 112) as well as in plant diseases.

In wheat leaves, *P. graminis* causes an increase in permeability to water and solutes (60). Similar effects were found after inoculation of tomato plants with *F. lycopersici* (113), or after application of culture filtrates of this

fungus (114) or of pure wilt toxins and antibiotics (115) to tomato cuttings. Fusaric acid (at certain concentrations) increases water permeability of protoplasts in normally nourished tomato plants but not in nitrogen deficient plants (116). Since nitrogen deficient plants are much less sensitive to fusaric acid as measured by external symptoms, increased permeability to water may be an important step in the reaction chain which finally leads to leaf necroses. Under varied experimental conditions, fusaric acid and its relatives do not necessarily increase water permeability. Depending on the chemical structure of the toxin, on the concentration applied, and on the species and metabolic state of the plant, water permeability may be increased or reduced; in part, these toxic actions may be overcome by certain metabolic inhibitors (117). An overall picture of these reactions cannot yet be drawn.

In the course of permeability increase in tomato cuttings treated with wilt toxins, considerable amounts of inorganic ions and amino acids are released from protoplasts and deposited on the leaf surface (118). The excretion starts prior to the formation of visible necrotic spots on the leaf and occurs also in leaf regions which do not become necrotic at all. Permeability decrease in connection with resistance has been mentioned earlier; in wheat races with a labile (mesothetic or x) reaction to *P. graminis*, the leaf regions with susceptible or resistant reaction may be correlated with local increase or decrease of permeability (119).

These changes in permeability may be expected to influence water flow in the plant. The problem has been studied with several wilt toxins. In the case of lycoramin, application of the toxin to tomato cuttings causes first a short, shock-like decrease in water uptake through the stem and water loss through transpiration. In a second phase, an increase in permeability causes a heavy water loss from the protoplasts and leads to an increased water flow in the plant. Water uptake and transpiration rate reach a maximum which may be higher than the values before application of the toxin. External disease symptoms appear in general at the end of this period. In a third phase, water uptake and water loss decrease again; protoplasts are heavily damaged and coagulated, and external symptoms become more and more severe (120). During the second and third phase water loss by transpiration is generally higher than water uptake. Few studies deal with transpiration rates of infected plants. In tomato plants inoculated with *F. lycopersici* (121) and in other diseased plants (122), transpiration rate during incubation is rather higher than in control plants; at about the time of appearance of the first symptoms decrease of transpiration begins and becomes more and more pronounced. As little is known about the cooperation of the factors which may be involved (rate of formation and action of toxins, progress of vessel plugging, etc.) the results are difficult to interpret. There seems, at least, to be no contradiction between *in vivo* and *in vitro* experiments; in both cases, final decrease in water flow begins rather late. For a final interpretation, however, more experimental studies are necessary.

Respiration.—In many plant diseases respiration of invaded and sur-

rounding tissues increases in the period following infection (123). Most of the increase can be attributed to an increased respiration of the host tissues under the influence of the parasite's toxins. The increase in respiration is explained by Allen (123) as an uncoupling of respiration from the energy requiring activities of the cells in a manner similar to the action of 2,4-dinitrophenol (124). In many cases, increase in respiration lasts only for a certain time and may end after a few days. In wheat plants inoculated with *Erysiphe graminis* respiration increases from the second through the sixth day and decreases slowly afterwards (125). In black rot of sweet potatoes, caused by *Ceratostomella fimbriata*, the respiration rate reaches a maximum after two or three days and decreases thereafter (126). In bean leaves inoculated with *Uromyces appendiculatus*, increase in respiration begins on the fifth or sixth day and leads to a maximum on the twelfth day which is followed by a decrease; the first external symptoms appear after eight or nine days (127). Corresponding to these results an increase in tissue temperatures which sooner or later is followed by a decrease has been observed in various diseases (128, 129, 130).

Virus infections may cause similar alterations in respiration (131, 132, 133). The reactions may, however, depend to a great extent on the metabolic state of the host plant. Respiration of tobacco plants during the first twenty hours after inoculation with tobacco mosaic virus is increased in winter but decreased in summer; spring and fall values are intermediate (134). What happens in this case is not yet clear. Seasonal changes in light intensity probably influence the metabolism of the host plants. If, according to Sempio (70), a relatively high respiration rate indicates an increased state of susceptibility, tobacco plants would be most susceptible to mosaic virus in winter. This does not seem contradictory to earlier results (133). It might also be mentioned in this connection that tomato plants are more sensitive to lycopersamin in winter than in summer (135). In a later stage of tobacco mosaic disease (three to six weeks after inoculation) no seasonal differences were found; respiration was normal in older leaves and decreased in young, actively growing leaves (136).

In certain diseases respiration follows a different pattern. The changes in respiration during the first period of crown gall development are not yet quite clear (137). Link & Goddard (138) found an increase in oxygen uptake (calculated on a fresh weight basis) whereas Klein (139) reported a slight decrease in oxygen uptake and an increase in anaerobic fermentation (calculated on a fresh weight or dry weight basis). During the growth phase oxygen uptake and anaerobic fermentation reach a maximum; later they slowly decrease but remain higher than controls until at least 44 days after infection (139). It might be interesting to compare crown gall tissue in detail with animal cancers where a disturbance of respiration and an increase in anaerobic fermentation has been found (140). Temperatures of crown gall tissues were found higher than those of controls six months after inoculation (129).

Few attempts have been made to isolate substances which increase res-

piration. Hellings (141) started purification of such a toxin from *Gibberella Saubinetii*. Ethylene produced by *Penicillium digitatum* contributes to the increased respiration of lemons (142, 123). In addition, a few antibiotics are known which have similar effects (123).

Among the wilt toxins, fusaric acid inhibits oxygen uptake and carbon dioxide formation by tomato leaf tissues to the same degree (143). In the endogenous respiration of yeast cells, fusaric acid and clavacin inhibit oxygen uptake but increase carbon dioxide formation (53, 144). Fusaric acid inhibits the respiration of leaf tissues from normal as well as nitrogen deficient tomato plants; decrease in disease symptoms in nitrogen deficient plants does not seem connected with changes in respiratory systems (116). Many questions remain open in this field.

Assimilation.—Sempio (70) reported a sharp increase of assimilation during the first two or three days after inoculation of wheat leaves with *E. graminis*. In the following days assimilation decreases and finally falls below control values. The author interprets the preponderance of assimilation over respiration in the earliest phase as the expression of metabolic resistance which is, however, overcome by the parasite subsequently. In wheat leaves inoculated with *Puccinia glumarum* assimilation remains normal during incubation and decreases afterwards; in resistant wheat the assimilation rate does not change (145). In general, increase of carbohydrates in early phases of infection is ascribed rather to accumulation from surrounding tissues than to increased assimilation (2, 3, 5).

Various physiological alterations.—Parasites may cause an increase of various enzymes (catalase, phosphatases), increase of auxins, accumulation of phosphates (146, 147), changes in nitrogen compounds etc. (2, 3, 5, 131, 133). In cotton plants inoculated with *F. vasinfectum* the ascorbic acid content during incubation is rather higher than in normal plants; with the appearance of the first symptoms ascorbic acid begins to disappear (148). A detailed picture of all these processes does not seem feasible at the moment.

General remarks.—Many observations mentioned in this section point to a considerable increase in metabolic activity during early disease phases. The various reactions involved in these processes decide the fate of cells and tissues. They may change cells into incipient tumor cells especially sensitive to auxin action (137, 149); they may cause in the cells a particular reactivity to wounding [wound tumor virus (150)], to secondary infections, etc. Other plants may answer the parasite's actions with defense reactions and find the way back to normal life. In many other cases, however, protoplasts become more and more damaged; morphological alterations of cells and tissues become visible and progress more and more, and the period of manifest disease begins.

CONCLUSIONS

Fundamental actions and reactions take place during the early phases of plant diseases. A few points only have been discussed here, and important problems of virus multiplication, toxic by-products of virus synthesis, al-

terations of the parasite under the influence of the host, etc., have been omitted. In most fields only scattered information is available which is often restricted to certain groups of diseases like rusts, mildews or Fusarium wilt. However, these studies have in general shown the importance of chemical interactions between the metabolisms of host and parasite during incubation. Further research in this direction may lead to a better knowledge of various theoretical and practical aspects of plant pathology.

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BATS, AND THEIR RELATION TO RABIES¹

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The disease caused by the rabies virus was described in some of the first medical writings recorded in history (1) and has continued ever since to challenge, and to provoke, the thoughts of students of medicine. For the most part the disease is seen in mammals of very many different species, but it has also been observed in birds (2, 3). The virus has also been reported to survive in the central nervous system of reptiles and turtles (4). Although the virus finds the tissues of many species suitable for growth, the animals recognized as most responsible for its transmission to new susceptible hosts are the carnivores, and these have received the most study through the years.

While susceptibility to infection and the period of incubation are subject to wide variation, the disease in most species is generally manifest by a period of restlessness followed by irritability and aggressiveness with a relatively late onset of paralysis, and death, which may terminate the disease at any stage. If the symptoms of irritability and aggressiveness (which may be quite violent) predominate, the disease is usually classified as the "furious type." If paralysis predominates, and this may be among the first symptoms observed, the disease is called the "paralytic" or "dumb type." A characteristic and impressive change in phonation, and hydrophobia may or may not accompany these symptoms. Among the usual mammals contracting the disease, the mortality rate is 100 per cent and death usually occurs in a few days after onset of symptoms. However, a few investigators have indicated the possibility of recovery (5).

Since the period of communicability is relatively quite short and the disease 100 per cent fatal, the manner of survival of the virus in nature has not been satisfactorily explained. This problem has been the subject of much study and it has been proposed that the extremely long incubation period could account for survival of the causative agent. However, the search for a host that could become a symptomless or recovered carrier continues, but none have been found among the usual hosts.

In the early part of the twentieth century certain species of another class of mammals of the order Chiroptera, containing the bats, were found to be responsible for the transmission of the rabies virus to other animals. The first observation of rabies in bats was made in Brazil. Later investigations soon demonstrated similar situations occurring in Argentina, Paraguay, Venezuela, Central America, Mexico, Trinidad, and Honduras. Until 1953, rabies in bats was known only in this part of the world. Further, the species of bats found responsible for the transmission of rabies to other animals and

¹ The survey of literature pertaining to this review was completed in January, 1956.

man were found only in this area. While these bats were shown to be true carriers of the virus and responsible for extensive epizootics among herbivores, it was difficult to see how this specialized situation could aid in the understanding of the basic life cycle of the rabies virus in other parts of the world, where the vampire or hemophagous bats did not exist. Then in 1953, and quite unexpectedly, the rabies virus was isolated from an insectivorous bat in Florida, and during the next two years from eleven other different species of insect-eating bats collected from eight widely-separated geographical areas of the United States. These findings stimulated avid interest throughout the world in the possible role this new intermediate host might play in the rabies problem. Recently, reports have been received from Germany and India indicating the possibility of similar findings.

SOME GENERAL INFORMATION CONCERNING BATS

The order Chiroptera is quite large, and our knowledge of the different kinds of these mammals is probably anything but complete. According to Sanborn (6), about 2000 species have been identified and these are grouped into 215 genera and 17 families. To this may be added sub-species and races. Bats have existed since the early Tertiary period (7) and have not changed too remarkably since. They are found around the world from the limits of the growth of trees in the north, to the most southern lands except for Antarctica, from sea level to as high as 13,000 feet, and in desert and swamp. In the tropics the greatest number of species are encountered, but this by no means should give the impression that the temperate zones do not have their populations in appreciable numbers. For example, within the United States three families comprising 16 genera and 65 species have been identified (6). Furthermore, the estimates of the number of bats to be found in some of our caves is in the millions (8). Evidence of the multitudes that have used the Carlsbad Caverns is contained in an account by Bailey, as cited by Allen (7), of the guano originally deposited in the outer part of the cavern. It was estimated that this deposit extended for a quarter of a mile, was a hundred yards wide and varied in depth to almost equal its width. These deposits were worked commercially for 15 years with about 1500 tons a year being harvested.

Bats vary in size from the largest having a wing spread of fifty inches down to the smallest of only five or six inches. They are equally different in their food habits and may roughly be classified into fruit, fish, flesh, and insect eaters as well as hemophagous, although the food habits of some species may be changed by training or under the pressure of hunger. A few species, especially those residing in the temperate zones, migrate as far as six to eight hundred miles to escape the rigors of winter (6). Their natural habitat is also quite varied; some dwell in trees, others in caves, and still others in buildings in close proximity to man and animals. From the epidemiologic and epizootic viewpoints, the intimacy of habitat between bats, humans and animals is a most intriguing observation.

Certain external features of bats may be used for classification. The length of the forearm has been very useful for quick identification in the field (9). However, more useful characteristics are found in the dental and cranial configurations (10), and these together with color and body contours, including the interdigital and interfemoral membranes, should be preserved if identification is desired.

The bats that inhabit Central and South America are quite varied as to habitat and food preferences and include species that eat insects, fruits, flesh and the vampire that prefers fresh blood. In the United States all the bats are insectivorous. Most species take their food on the wing, but some capture grubs on the ground. A few visit nocturnal flowers in search of insects and perhaps pollen.

The vampire bat has been surrounded by much folk-lore and the stories relating to its blood lapping propensities have grown and become about as enlarged as the stories of its supernatural powers. In any event, many have been found to be infected with the virus of rabies. The vampire bat, because of its strict hemophagous habit, has become the most important vector of rabies in both Central and South America, the area to which it is confined. Ever since Columbus discovered the Island of Trinidad in 1498, it has been known that hemophagous bats exist in the Western Hemisphere, and the conquistadores invading the eastern shores of Yucatan were the victims of attacks by bats that inflicted not only injury to their beasts of burden, but to the men themselves (12, 17). Other early explorers also described their encounters with bats (13) and Darwin (14), while in Chile in 1832, captured a vampire as it was feeding on his horse's back.

There are three genera in the family *Desmodontidae*, that contain the vampires (7, 15, 17), but the species *Desmodus rotundus murinus*, Wagner (1840), is the vector of greatest importance in the transmission of the rabies virus (11). Its geographical distribution extends from Chile and Argentina in the south to within 100 miles or less of the United States-Mexican border (11). There are many good descriptions of the method they use to obtain blood from their victims (7, 11, 15, 16, 17). They are quite uncanny in sensing when their victim is asleep, at which time they light a little distance away, crawl to the site of choice and make their incision, often without disturbing the host. The bite is shallow, crater shaped, sharply circumscribed, and about four mm. in diameter. The bat laps up the blood as it fills the crater. Bleeding is aided by an anticoagulant in the bat's saliva (38) and may stop in five to ten minutes, or continue longer. However, the same or another vampire bat may return, lift the scab and feed again from the same site. It has been observed that they will consume as much as 50 ml. of blood at one feeding (17, 18).

The natives inhabiting the Island of Tabago are reported to say that before horses and cattle were brought to the island the vampires fed on sea fowl (19). Malaga-Alba (11) reports that he has observed them feeding on chickens. With the approach of darkness the vampire flies in search of his

blood meal, and when finished retires for a period of digestion after which he may return before dawn for another feeding. He usually sleeps during the day, but not always. It is obvious that the specialized feeding habits of this species provide ample opportunity for the transmission of the rabies virus not only through the initiation of the incision, but during the lapping-up of the blood. More evidence of the proficiency of these mammals as carriers and vectors will be presented along with the investigations into bat transmitted rabies by various workers in Central and South America.

The outstanding characteristic of bat transmitted rabies, in all species of animals and in humans, is the early onset of a rapidly ascending, or in some cases descending, paralysis. In the majority of animals this initiates in the hind quarters. For this reason the disease has been designated by various names in different countries, all of which refer to the weakness of the hindquarters. Thus, in Brazil the disease was first called *mal de caderas* or *peste dos cadeiras* (20); in Paraguay, to distinguish it from trypanosomiasis and because of the hemorrhagic appearance of the meninges, *pasteurellosis paresiante del Paraguay* (22). In Argentina it was called *rabia parisiente* as well as *mal de caderas en los vacunos* (23). In Central America and Mexico, according to Malaga-Alba (11), it has been designated by the following terms: *renguera*, *derrengue*, *tronchado*, or *derriengue* (24, 25). In Trinidad the disease in cattle was thought to be botulism. The human disease was diagnosed as an acute ascending myelitis, thought to be caused by an aberrant strain of the poliomyelitis virus (26).

INVESTIGATIONS IN SOUTH AMERICA

Brazil.—Between the years 1906 and 1908 a paralytic disease of cattle, horses, and mules appeared in the state of Santa Catharina in southern Brazil. It appeared first in a relatively restricted area between the mountains and the sea, but in epizootic proportions. The disease was first observed in the bovine and was thought to be rinderpest (27) and some time and effort were spent on an immunization program against this disease. Before long the disease was observed in horses and was called *mal de caderas*, thus confusing it with trypanosomiasis. Some cases exhibited signs of cutaneous itching and for a time it was thought the malady might be Aujeszky's Disease, which was also occurring in the area.

In 1911, Carini (20) of Sao Paulo came to investigate the disease. In his examinations he found Negri bodies in brain tissues from bovines dying of the disease and succeeded in producing the paralytic disease in rabbits with the same material. However, he could not demonstrate Negri bodies in the first passage rabbits, even though they were present in the bovine tissue inoculum. Nevertheless, he diagnosed the condition as being caused by the rabies virus. He described the disease as progressing quickly from early symptoms of excitement to paralysis, although from the beginning incoordination of movement especially in the hindquarters was observed. The incoordination would increase until the animal would stagger and fall.

Paralysis was widespread and the animal would die in three to five days. Carini noticed that canine rabies was quite rare and seemed to bear no relation to the occurrence of the paralytic disease in horses and cattle. He suggested that this pointed to the spread of the paralytic disease by some wild animal. It was called to his attention by the people of St. Pedro d'Alcantara, and by the inhabitants of other affected districts, that bats had been observed flying about in broad daylight and actually biting cattle as they grazed in the pastures. Further, these animals later contracted the disease and died. However, he was unable to obtain any of the bats at that time for examination.

With the knowledge that this disease was paralytic rabies, a strict and extensive program of dog control was instituted with full police powers to implement it, and in six months of 1912 over 6800 dogs were destroyed. A very strict ban on importation of all canines was also put into effect. In spite of these measures, the epizootic continued to spread among cattle, horses, and mules. It was further noticed that the greatest number of cases occurred near the sparsely settled forest areas and that in these areas there was no particular difference in incidence on either side of swiftly flowing streams. Contrarily, there were fewer cases in the more densely populated areas. All these facts again pointed to the absence of any connection between the cattle disease and dogs, and after a time the campaign against dogs was discontinued. The suspicion that a flying sylvan animal was responsible for the dissemination of the virus was again emphasized.

By 1913, the disease had spread to the settlement of Blumenau and attained very serious proportions, some 30 per cent of the cattle and 15 per cent of the horses succumbing to it. Two German veterinary workers were engaged to investigate the epizootic. Dr. Haupt (28) arrived in 1914 and Dr. Rehaag (28) in 1916. They soon confirmed the diagnosis of Carini, that the disease in cattle was definitely a paralytic form of rabies. They also emphasized the lack of correlation between canine rabies and the cattle disease. Their attention was soon drawn to the peculiar occurrence of fighting, shrieking bats flying about in the day time, and in their opinion such unusual aggressiveness probably indicated disease.

The inhabitants of the areas were also convinced that these bats were spreading the disease, and brought some in to be studied. These were identified as *Phyllostoma superciliatum*, Burmeister (1884), a fruit-eating bat. In the stomach of two of these, partially digested blood was identified. On July 1, 1914, a nose-leaf bat was observed biting an eight-day-old calf which until this time had been kept in a bat proof stable. Thereafter this animal was protected from further bat bites, and 27 days later developed typical paralytic rabies. Attempts to ascertain the presence of the rabies virus in the bat brain were not undertaken. It was not until 1916 that Rehaag succeeded in isolating the rabies virus from the medulla of another *Phyllostoma* bat that had been observed biting a bovine. This virus induced paralytic rabies in a guinea pig and a rabbit. Sections of the brain of both

the guinea pig and rabbit contained Negri bodies. On the strength of this evidence Haupt & Rehaag (28) concluded that the virus of paralytic rabies, which had caused the death of so many animals in South Brazil from 1915 to 1918, was spread by bats. The opinion of Haupt & Rehaag was further strengthened when it was observed that preventing bats from biting cattle effectively reduced the incidence of the disease. Kraus & Duran (29) also agreed that bats were probably responsible for the spread of paralytic rabies.

Alves De Souza (30, 31), as cited by Pawan (38) and Carneiro (33), also determined that the disease of cattle occurring in the state of Rio Grande do Sul (located on the coast to the south of Santa Catharina) and in the State of Matto Grosso (an interior state to the north of Santa Catharina) was paralytic rabies. However, he was not able to transmit the disease to guinea pigs and rabbits by allowing *Phyllostoma* bats to feed on them.

De Queiroz Lima (32) and Torres & De Queiroz Lima (84, 85, 86) undertook a series of experiments to ascertain whether or not the vampire bat could be artificially infected with rabies and what the course of the disease would be in this animal. They were able to show that *Desmodus* can artificially be infected with the bovine virus, and that the experimentally infected animal can infect other bats by natural means. They further demonstrated that these naturally infected bats can transmit the virus to cattle during feeding for a period of 110 days, and that some of the bats capable of transmitting the rabies virus apparently were quite healthy. Later, while in the state of Santa Catharina, De Queiroz Lima (32) isolated the rabies virus from two vampire bats that were collected in epizootic areas. Torres & De Queiroz Lima concluded that paralytic rabies of the herbivores is carried by the hemophagous Chiroptera which form the Desmodontidae Family, and especially by *D. rotundus*, the most numerous species in the family.

Epizootics of bovine paralytic rabies have continued to make their appearance in Brazil. In the course of these episodes untold thousands of cattle have perished, but no relation to canine rabies has been observed. Carneiro (33, 83) states that successful preventive anti-rabies vaccination of bovines was first demonstrated in the Brazilian states of Matto Grosso, Espirito Santo, and Santa Catharina and is still employed. It is the only practical means of controlling the disease. No cases of human paralytic rabies have been reported in Brazil.

Paraguay.—During September of 1928, the outlying estancias reported serious outbreaks of *mal de caderas* in the bovine species. In certain areas 25 to 30 per cent of the herds were lost. Lindsay (22) reported that in spite of segregation of herds, the burning of carcasses, and disinfection of premises the epizootic continued. He stated that a fairly regular symptomatology was encountered in the bovine. Earliest signs of onset of the disease consisted of anorexia, roughened hair, general tremor, and dragging of the hind legs. Often this would be coupled with a noticeable weakness in the forelegs and diarrhea or constipation, the latter seen more frequently in the milk cow.

Over the next two or three days paralysis would progress until the animal would fall. Death occurred during the next two or three days. Post mortem examination revealed congestion and hemorrhages of the meninges, but this was not thought to be sufficient to account for the manifestations of disease. Rather, an intoxication was presumed, possibly due to a poisonous plant (27). Because of the clinico-pathological findings the name *Pasteurellosis Paresiante del Paraguay* was suggested to distinguish it from true *mal de caderas* in horses and from other hemorrhagic diseases. Rosenbush (35) in his studies of the disease in Paraguay was of the opinion that the rabies virus was the causative agent and sent some material to Remlinger and Bailly of the Pasteur Institute who confirmed his impression. Migone & Pena (21) also believed the disease was caused by an agent very closely allied to the rabies virus, but differing in virulence. They sent material from a bovine case to Professor Kraus in Santiago, Chile. He too diagnosed the disease as rabies and noted that rabbits, sheep, dogs, and guinea pigs all were susceptible. Lindsay also reported that bats were caught in an affected area and Negri bodies were found in their brains. However, because of the tremendous size of the estancias and the remoteness of the cattle population an extensive investigation of bats could not be made. Two severe outbreaks of canine rabies were reported by Lindsay (22) in Paraguay. The first epizootic, in 1926-27, just preceded the cattle disease while the second, occurring in 1931, coincided with the last phases of the bovine epizootic. These two outbreaks of rabies in the dogs of Paraguay, while having a certain temporal relationship to the bovine epizootic, exhibited little resemblance otherwise. Neither in the geographic distribution of the cases, nor in symptomatology were the outbreaks of rabies in the two species similar.

The sylvan origin of the paralytic disease in cattle is well illustrated in Paraguay as the affected cattle were mainly to be found in the estancias and often in their remote parts near forested areas. The urban herbivore population was little affected. There were no cases of human paralytic rabies associated with the epizootic in cattle in Paraguay.

Other South American countries.—The disease known as *mal de caderas de bovinos* and shown to exhibit a spreading type of paralysis, most often affecting the hind quarters first and rapidly ascending, was found to be present in Argentina (23), British Guiana (27), and Venezuela (36, 37). The rabies virus was shown to be the causative agent of the disease in these countries, and the characteristics of the epizootics were similar to those found in Brazil. In Argentina, the periods of greatest incidence were associated with the rainy season. In Venezuela, the etiological agent of the disease was identified in 1938, although the disease itself had been seen much earlier (16, 27, 34, 38). Experiments with the Venezuelan virus (37) indicated that cattle and goats were very susceptible and the pig and the dog relatively resistant to the virus, while for laboratory investigations the white mouse seemed to be the host of choice. Observations of the course of the disease in larger animals always revealed an early onset of paralysis. The paralysis progressed

rapidly, but paralysis of the jaws in the large animals or of dogs was never observed. Negri bodies were found in about 70 per cent of the affected dogs, and they were relatively small in size.

In Venezuela, prevention of the disease in the bovine was successful by immunization with a vaccine produced in calf brain from two strains of the virus isolated in the country. Vaccine made from one of these strains (Bollivar) conferred on mice better protection against the homologue virus than a vaccine produced from a virus strain obtained from the Pasteur Institute (37).

TRINIDAD

Trinidad is a small island of some 2000 square miles located about twelve miles off the coast of Venezuela. Between its north shore and Venezuela are some small islands, while the southern part is separated from the mainland by a broad expanse of sea. The island has been known to be inhabited by bats since the time of Columbus, and there are official records of these bats biting humans since 1870 (38). Canine rabies was known on the island, but strict regulation of dogs was put into effect and canine rabies gradually came under control. The last reported case of rabies in a dog occurred in 1914. The last case of rabies in humans acquired from the bite of a dog was reported in 1912.

In July 1925, it was reported to the authorities that young heifers grazing in a pasture near St. Anns were dying of a peculiar disease. St. Anns is a suburb of Port of Spain, the capital city of Trinidad located in the north west portion of the island, which is the area closest to Venezuela. The disease soon appeared on two Government pastures (27) in the Port of Spain area, the St. Anns and the Queen's Park savannahs. These were public pastures used by the dairy operators of the vicinity. The incidence of disease among these herds was about 20 per cent. It was observed that all the affected animals were out of doors day and night, but animals in stables in the immediate vicinity remained healthy. Obviously there was something abnormal in the environment of the savannahs, and the owners were advised to remove the animals to their own premises. Some of these animals which left the public pastures in apparent good health would fall victims to the disease as long as one month later. Animals kept stabled at the owners' premises remained healthy even though in close contact with those that became ill. Meanwhile the owners were carefully questioned regarding any changes in husbandry and the grazing land was diligently searched for poisonous plants, all to no avail. The disease then made its appearance in another public pasture about one mile west of the first area and then subsided and appeared to have died out. Some months later towards the end of 1925 and early in 1926, it appeared about four miles to the west. Again the only animals affected were those kept outside during the night. The outbreak was soon over and there were only a few sporadic cases during 1926.

In 1927, it reappeared in the area, only now there were reports of a few

stall-fed animals contracting the disease. These stables are open sheds with only a roof overhead. There were cases also seen in other locations in the general area during 1927, all within a radius of 28 miles. In 1928, except for a few cases during the rainy season, the disease seemed to be disappearing. Suddenly with the start of the rainy season in 1929, it flared up causing heavy losses in livestock in the Siparia district 55 miles to the south. From this area it spread in all directions until by 1934 it had been seen in all parts of the island, except beyond the northern range of mountains. During 1929, 1930, and 1931 over a thousand cases a year occurred, 90 per cent in cattle, but also in horses, mules, donkeys, goats, sheep and pigs.

The first sign of the disease in the bovine was a tendency for the animal to graze apart from the rest of the herd. Attempts to drive the animal resulted in a slow side to side movement of the head, grinding of the teeth and some salivation. Tremors and incoordination of movement in the hind quarters became more pronounced on exertion. Anorexia, increased salivation due to an apparent inability to swallow, and posterior paralysis developed. The animal might be irritable in the early stages, although attempts to charge were futile because of incoordination and paralysis. In three to five days the progression of paralysis resulted in death by respiratory failure. In a small percentage of cases onset of paralysis was in the front legs. Temperature was usually normal and pulse rate increased, but weak (27). The disease in horses resembled that in cattle, except that horses frequently showed more excitement in the early stages and were reported to bite themselves. Pigs, sheep and goats exhibited an early and progressive onset of paralysis. Gross pathological findings were few, the chief finding being congestion of the membranes covering the central nervous system. Microscopically, perivascular infiltration and granular degeneration of the nerve cells were usually seen, while foci of glial proliferation were frequently, but not always, encountered (16, 27, 39).

Also in 1929, in the vicinity of Siparia, a peculiar disease involving 13 humans appeared (26, 34, 38). It was characterized by an ascending myelitis and a 100 per cent mortality rate. The human disease was tentatively thought to be poliomyelitis, although in a somewhat aberrant form. De Vertuil & Urich (34) described the disease in the following terms: an elevation of temperature of two or three degrees and sensory disturbances of tingling, numbness and burning, followed by paralysis of the affected limb, that spread. Retention of urine, constipation, paralysis of the arms, salivation and respiratory failure marked the course upward from the legs. Initiation of symptoms in an upper extremity, while marked by spreading involvement and respiratory failure, often left the knee reflexes unimpaired. The histological picture, described by Hurst & Pawan (26, 39), presented widespread nerve cell destruction and microglial proliferation, affecting both the anterior and posterior horns of the spinal cord and to a lesser and more variable extent the nerve cells of the higher centers. Inflammatory changes and perivascular cuffing were quite variable and sometimes minimal.

Pawan (26), in examining sections of brain of a human case, and of rhesus monkeys inoculated with brain tissue from human cases, observed inclusion bodies that resembled Negri bodies. He forwarded material to Hurst (26) of the Lister Institute and to Flexner at the Rockefeller Institute; both confirmed his diagnosis that the outbreak of human disease was rabies. Material from bovine cases was then found to contain Negri bodies and the diagnosis of rabies was again confirmed by Hurst & Flexner. Hurst emphasized the similarity between the Trinidad situation and the earlier episode in Brazil in which the vector, while unknown, was suspected to be the bat. Tissues from the Trinidad cases were subsequently sent to Andrews, at the Webbridge Laboratory in England, to Rous, of the Pasteur Institute, and to Finzi, of Milan, Italy, all of whom agreed that the disease was rabies.

Acting on the suggestion that bats might be infected with the rabies virus, Pawan (38) collected 157 bats, not identified as to species, and 52 fruit eaters (*Artibeus*). These were examined for Negri bodies and inoculated into rabbits during the period August 1 to September 8, 1931. In no case was evidence of the rabies virus found. However on September 10, 1931, a leaf-nose (*Artibeus planirostris trinitatis*) fruit-eating bat was caught flying in a chemist's shop at 11 a.m., and Negri bodies were found in sections of its hippocampus. Brain material from this bat was inoculated intracerebrally into a rabbit, inducing paralysis of the hindlegs on the twelfth day and death on the thirteenth day post-inoculation. The hippocampus of the rabbit also contained Negri bodies (38). Attention was then given to further investigating the role bats might play in the paralytic rabies of both animals and man on Trinidad. During the next few years, bats were caught from both endemic and nonendemic areas. Almost four per cent of these were found to have either Negri bodies in their brains or to induce paralytic rabies in animals upon inoculation (38). Among the bats that were positive there were 64 *desmodus*, 4 *artibeus*, and 1 *hemiderma*. By far the greatest percentage of these found positive were captured in areas in which the disease in animals was endemic (34).

Experiments conducted by Pawan (40) with vampire bats artificially inoculated with a strain of the rabies virus isolated from a human case, and with those acquiring the infection naturally, demonstrated that infection with the rabies virus in these mammals may express itself in either the furious or paralytic type of the disease, or may be symptomless. The bats meanwhile can transmit the virus by their bites. Furthermore, they may recover from the furious stages of the disease and continue to be capable of transmitting the virus for prolonged periods of time. Later experiments with both artificially and naturally infected *A. planirostris* (the leaf-nosed, fruit-eating bat) revealed the same capabilities (41). Rabbits, dogs, guinea pigs, and calves were injected with the brain material from the peculiarly acting vampires. All of these that became ill developed paralytic rabies. One vampire that remained in good health while in captivity for five and one-half months, still had the virus of rabies in its brain. One monkey inoculated with

brain tissue, and one monkey with the salivary gland tissue of a *D. rotundus* bat developed furious rabies. However, tissue from the medulla of these monkeys induced paralytic rabies in four calves, one dog, and a rabbit (38, 40, 41).

Surveys conducted in the field brought to light the fact that the inhabitants of the area, who were quite familiar with the normal habits of the vampire bat, had observed that these bats were flying about in broad daylight, fighting with one another and viciously attacking humans and animals. They also noticed that the animals that were bitten were those that became ill, and they were firmly convinced that bats were spreading the disease. To obtain definite information on this point, a field experiment in three epizootic areas was conducted (38). This consisted of bat-proofing certain stables and not others, together with close observation of the animals. The results were most revealing. The animals protected from bats remained healthy while all those not so protected were bitten by bats, and contracted the disease.

Cross-protection tests using rabbits immunized with virus fixe and with the bat virus and then challenged with the homologous and heterologous strains of the virus were made. The results of these tests revealed better protection conferred by the virus fixe antigen against the bat virus than by the virus vaccine against virus fixe.

Attempts to illustrate the relationship of bats to the human cases of this paralytic form of rabies were made (34, 38). It became obvious after questioning 47 patients that all but one remembered distinctly being bitten by bats. This could often be confirmed by other members of the family who had aided in chasing the bat away. The one exception to this was a man who could not remember any recent bat bites. All these patients with history of bat bite, except one, developed their first symptoms in the same extremity in which they were bitten. The exception was bitten on a toe of the right foot while symptoms first appeared in the right upper arm. Certain other epidemiologic features seen both in the human and the animal disease further strengthened the hypothesis that both were disseminated by bats, especially the vampire. De Vertuil & Urich (34) list these similarities as follows: (a) The duration of the outbreaks were quite similar and both seemed to occur sporadically between outbreaks and then flare up again. (b) In both the peak incidence was associated with the rainy season and both were seen more often in populations presenting greater opportunity for attack by bats. (c) The cattle epizootics always preceded the human outbreaks. (d) The incubation periods of the two hosts varied within corresponding limits. (e) The virus isolated from humans was similar to that isolated from animals in its ability to cause paralytic rabies in rabbits. (f) An obvious difference was the relative confinement of the human cases on Trinidad in relation to the many areas in which cattle were affected. This point was rationalized on the basis that animals are the most convenient source of blood for the vampire and when these are protected from bats or reduced in number, humans are attacked.

Methods for the control of bats have been developed in Trinidad. Those relying on bat proofing of structure, repelling them with night lights and destruction of their colonies have their uses in certain specialized situations but the most effective means seems to be immunization with virus fixe or with an homologous strain as practiced elsewhere in South America. Pawan (49) reports that paralytic rabies continues to be observed in the animals of Trinidad, but sporadically and not in large epizootics. Eighty-nine human deaths due to this disease have occurred on the island, the last in 1937.

CENTRAL AMERICA

There are many reports of an ascending paralytic disease of cattle occurring throughout Central America. In 1949-50 a severe epidemic appeared in the neighborhood of La Cieba, Honduras (43), characterized by a progressive ascending paralysis. Paralysis usually started in the hindquarters and early loss of the sensory reflexes was noticed. The disease would terminate by death, four or five days after onset. In the early stages of the disease there would be licking paroxysms, a tendency to turn the head to one side and efforts to circle. Various suggestions were considered as the cause of death; among these were Aujeszky's Disease, listeriosis, botulism, and poisonings. Brain tissue from cattle dying of the disease was sent to the Louisiana Livestock Sanitary Board, where Negri bodies were found. Later isolations were made in mice at the Virus and Rickettsial Laboratory, Communicable Disease Center, United States Public Health Service. The possibility of the disease being transmitted by the vampire bat was immediately recognized. Investigation showed that there were many vampire bats in the area, mainly *D. rotundus*. Further, evidence that these bats were feeding on the cattle and horses was frequently encountered, and the incidence of the disease was highest in those areas bordering on the forests, where vampire bats were most numerous. The inhabitants of the areas, however, had not noticed any peculiar behavior among these bats, either in fighting among themselves or attacking animals by daylight. Fifty-four bats were trapped, but attempts to isolate the rabies virus were unsuccessful. Schroeder and co-authors (44) reported that the rabies virus was isolated from six of seven bovine brains submitted to the Lederle Laboratories, Pearl River, New York.

In Honduras, Costa Rica, and Guatemala prevention of the disease was attempted by an extensive anti-rabies immunization program using a live attenuated virus. This program was as successful as in other areas of Latin America, for no cases of rabies occurred in the vaccinated animals during an observation period of from six months to one year, while the high incidence of disease continued on the same ranches among the non-vaccinated (44). The disease was not reported as occurring in humans.

MEXICO

A disease of herbivores has existed in certain parts of Mexico for many years. The disease is called derriengue, referring to an injury of the spine,

for it is characterized by an abrupt appearance of paralysis in the hind quarters. Rapid extension of this paralysis develops and death usually occurs in three to four days after onset. Johnson (45) reported that epizootics occurred in areas with a periodicity of one to two years, and during their course as many as fifty per cent of the cattle in the area may be destroyed. Early references only noted the disease in the warm humid coastal areas of the country, but in later years derriengue has been observed in other parts of Mexico. At the present time, according to Malaga-Alba (46), it has been reported from all the States of the Pacific Coast, from all but one of the Gulf Coast States, and from eight of the more inland States. A total of 22 of the 32 States and Territories of Mexico have reported the disease. It has been occasionally observed in the States of the high central plateau, although not in epizootic form. The distribution seems to be related in its spread to the environments found in the canyons that lead inland from the sea and retain the warm humid characteristics of the coastal areas.

Giron (25, 47) reports that in 1932, a virus was isolated from cattle that was considered to be the etiologic agent of derriengue. The experimental disease induced with this virus, it was noted, resembled rabies, but canine rabies was unknown during many of the epizootics of the disease in cattle. In addition, vampire bats had not been identified in areas where the disease was prevalent. Therefore, studies to relate this virus to the rabies virus were not attempted, and the identity of the agent remained unknown. Camargo (48), in 1935, reported that the virus of derriengue was isolated from the brain tissue of a horse and was shown to be distinct from the virus of equine encephalomyelitis.

In 1943, during an outbreak of derriengue, the brain tissue from a fatal bovine case was examined at the Rockefeller Institute for Medical Research, and rabies virus was isolated (45). Subsequently, the virus isolated in 1932 and considered to be the causative agent of derriengue, and one isolated from a typical case in 1941, were all identified as rabies by neutralization with known rabies antiserum. The 1943 isolate was further identified with the rabies virus by cross complement-fixation and cross-protection tests, and it was demonstrated to be relatively avirulent for dogs. When rabies was induced in dogs following intramuscular inoculation (45) with this virus it was characterized by an abrupt onset of incoordination and paralysis without the development of Negri bodies. Johnson (45) reported that one dog injected intracerebrally with 0.25 ml. of a ten per cent suspension of brain tissue of the fourth mouse passage of the 1943 isolate, developed paralysis on the nineteenth day after inoculation. This paralysis progressed until the third day of illness when the animal was completely prostrate. This dog recovered, and eleven months later when it was sacrificed, exhibited only some weakness in the hind legs. Neutralization tests using pre- and post-inoculation blood sera revealed the appearance, during this episode, of neutralizing antibody for the rabies virus.

In 1944, an effort was made to investigate the possibility that derriengue

was of vampire bat origin. The study was undertaken by Johnson (45) in seven localities in the States of Michoacan and Jalisco. In these areas the incidence of disease has been of epizootic proportions within the previous six months. In one area, a cow paralyzed with derriengue was sacrificed, and the rabies virus isolated from her brain and salivary glands. Mice inoculated with either the brain or salivary glands became paralyzed, and Negri bodies were found in their brains. Vampire bats, *D. rotundus*, were found in a cave nearby. A pool of the salivary glands, and a pool of the brains from eight of these bats were inoculated into mice and the virus of rabies isolated from each. Negri bodies were found in the brains of the first passage mice, but could not be demonstrated in the impression smears of the bat brains. Paralytic rabies was always induced in mice and guinea pigs with this virus. Antigenic studies revealed a close relationship with virus fixe as exhibited by cross-neutralization and by cross-complement fixation tests.

In the other six locations the outbreaks of derriengue had subsided before the survey started. While vampire bats were found in all six areas, attempts to isolate the rabies virus from them failed.

There was no evidence that derriengue in these areas was transmitted by the canine species. During 1944, there were no reported cases of rabies in dogs in the locale of the study, although it had been encountered in the past (45). In 1951, in the village of El Platanito, Malaga-Alba (11) reported that a furious bat attacked two neighboring families in the early hours before dawn. Ten of the thirteen members of the two families were bitten after repeated attacks. Subsequently, four of the children and one adult died of paralytic rabies. In June of the same year, in another state in which derriengue is enzootic, three of seven people attacked by a vampire bat also died of the same disease.

The studies of the distribution of derriengue and the observations on the range of the vampire bat, *D. rotundus*, in Mexico have been followed with great interest. It is not known whether the disease is spreading or is just becoming more widely recognized and reported. Recent studies of the distribution of *D. rotundus* have indicated a much greater range than heretofore recognized, and paralytic rabies has been diagnosed in three of the five States, Sonora, Tamaulipas, and Chihuahua (50) that border on the United States. It has also been reported that probable resting places of vampire bats have tentatively been identified along the Rio Grande (51).

THE UNITED STATES

In the United States, prior to 1953, the problem of rabies transmitted by bats was viewed with academic interest. A few investigators expressed concern, due mainly to seemingly isolated incidents and observations that had occurred. However, the eventual establishment of the vampire bat in the United States, while considered a remote possibility in certain areas having the environmental requirements, was not thought to be very probable. Insectivorous bats, the only kind known in the United States, were not

known to be infected with the rabies virus. However, Reagan & Brueckner (52) had shown experimentally that two species of our insect-eating bats were susceptible to infection with a canine strain of the virus.

In 1952, a survey was conducted in southern California by the Pan American Sanitary Bureau to investigate the possibility that vampires had become established in the area. While no vampires were encountered, certain circumstantial evidence obtained in the study was intriguing, but hardly conclusive. Horses and cattle with paralytic rabies had been reported in the area and wounds suggesting vampire bites had been found on the ears of other horses (50). In May of 1945, the San Diego Zoo lost a giraffe that had exhibited symptoms resembling an encephalitis (42) and the rabies virus was isolated from its brain (54). This expensive animal was protected by a woven wire fence through which no dog could pass, and rabid rats had never been found in the area. However, Schroeder (42) reported that nectar-eating bats had appeared in the area about that time. This interesting observation describes them as on an adventurous flight from Mexico (42). Further reports indicated (56) that a member of the United States Fish and Wildlife Service had seen a bat at close range which he thought was a vampire. There also were complaints from ranchers in northern Baja California of bats damaging the teats of nursing sows, a favorite site of attack by vampires. A little further north in 1944, a horse had died after an illness lasting two days. While a preliminary diagnosis of encephalomyelitis was entertained, nervousness and a definite muscular paralysis of the throat was thought peculiar (53). Subsequently, from the brain of this horse the rabies virus was isolated. Hammon (54) noted that this virus and the one isolated from the giraffe, behaved in experimental animals like strains isolated from vampire bats.

Sulkin & Greve (55) described an incident occurring in Texas in 1951, in which a woman who had picked up a sick bat on a roadside was bitten on the forearm. She became ill sixteen days later and stated that the arm hurt. Later, complete paralysis of the arm developed. Soon she was unable to swallow, became comatose and died. A tentative diagnosis of bulbar poliomyelitis was made. Histopathological findings revealed encephalomyelitis with demonstrable Negri bodies in the central motor neurons. It was concluded that this was a case of bat transmitted rabies. Unfortunately, the bat was not available for examination.

Florida.—In West Central Florida on the morning of June 23, 1953, a 7-year-old boy was searching the shrubbery alongside a field for a lost ball, when suddenly he was attacked by a bat; this was reported by Venters *et al.* (57) and Scatterday (58). The bat bit him several times and then remained firmly attached to the upper pectoral region. The boy ran to his mother who knocked the bat to the ground and the father killed it. The owner of the ranch, knowing of the vampire bat rabies problem in Mexico, submitted the bat for examination to the Tampa Regional Laboratory of the Florida State Board of Health. Numerous typical Negri bodies were observed in its brain,

and mouse inoculation proved the presence of rabies virus. This diagnosis was confirmed by the Virus and Rickettsial Laboratory, Public Health Service, Communicable Disease Center, Montgomery, Alabama. The bat had inflicted several discrete, puncture-type wounds deep enough to bleed on the shoulder of the boy. Twenty-eight hours after the attack, he developed symptoms of severe shock, with nausea and vomiting, but after intravenous therapy with glucose and saline this episode passed. Immunization with the Semple vaccine was instituted and the boy has evidenced no symptoms of rabies.

The bat was identified as a lactating Florida yellow bat, *Dasypterus floridanus*. This free-living, migratory, insectivorous species is quite numerous in Florida. It, or the eastern yellow bat, *Dasypterus intermedius*, which is practically indistinguishable from the Florida yellow bat, ranges along the Gulf coast and into Mexico, where it has been identified in the same areas as the vampire bat (59).

On confirmation of the diagnosis of rabies, the Florida State Board of Health undertook a survey to determine the prevalence of rabies in bats (57, 58). During July, August, and September, 1953, 208 bats of 3 species were collected within 35 miles of where the boy was attacked. All of these were free-living, insectivorous bats and apparently normal. The rabies virus was isolated from 5 of 138 yellow bats (*D. floridanus*) and from one of 61 Seminole bats (*Lasiurus seminola*). Four of the positive bats were collected on the ranch where the boy was bitten. Of seven positive bats, six revealed Negri bodies in their brains while in the seventh, atypical inclusions were seen. Negri bodies were usually seen on first mouse passage. In addition to these free-living bats, 300 colonial bats were examined in 1953. In the brain of one of these, a little Florida brown bat (*Myotis austroriparius*), collected in a cave in northwest Florida, atypical inclusions were found, and the rabies virus was isolated (59, 60).

An investigation to determine the source of the infection in the insectivorous bats of Florida led to questioning of workers loading and unloading bananas shipped to the port of Tampa. None of these men had ever seen a stowaway bat, although mice, opossums, snakes, and spiders had all been seen. Inquiry among United States Customs, Plant, and Animal Quarantine officers was also fruitless (61). In the county in which the boy had been bitten by the bat, one dog, one raccoon and one cow were proven rabid in 1953. However, since 1951, there had been an increase in sporadic unexplained cases of rabies in the wild life of Florida, particularly in raccoons.

Pennsylvania.—Kough (62) and Witte (63) reported that on the evening of September 29, 1953, a bat suddenly landed on the upper arm of a woman who was standing near a lake watching her husband feed water fowl. The bat bit her, leaving three distinct tooth marks on her upper arm, and scratches on both the shoulder and arm. She seized the bat, threw it against a fence and stunned it, whereupon her husband captured it. The woman proceeded directly to the doctor where the wounds were cauterized and the Semple

anti-rabies immunizations started. She has remained well. The husband of the victim, being a naturalist, recognized the behavior of the bat as abnormal. He submitted the bat to the Pennsylvania Bureau of Animal Industry for examination. Impression smears of sections of its brain contained typical Negri bodies. This was confirmed by the Pennsylvania Department of Health and at the Virus and Rickettsial Laboratory of the Communicable Disease Center. Two rabbits were intracranially inoculated with a suspension of the brain material from this bat and they died of rabies, one after 24 days and the other after 27 days. Subsequently, confirmation of the identity of the virus by serum neutralization was obtained.

Although the bat was destroyed before identification, all who had seen it later identified it as a hoary bat (*Lasiurus cinereus*). The scars on the woman's arm would fit the dental measurements of this species (63). The hoary bat migrates south as far as central Mexico to escape the cold winter months. It is an insectivorous, free-living bat found distributed over most of the United States in the warmer months. It is a relative large animal with teeth capable of inflicting appreciable wounds. A survey was instituted to collect and examine bats for rabies in Pennsylvania. However, due to the onset of cold weather, only seven bats were collected and these were all negative by impression smear.

Texas.—Rabies has long been endemic in certain parts of Texas in both domestic and wild animals. In the last ten years there has been an increased incidence in bovines, foxes and skunks (64). The Texas State Health Department, during November and December 1953, collected two hundred bats in central and south central Texas and examined them for rabies. Irons *et al.* (64), and Sullivan *et al.* (65) report that these included 151 Mexican freetails (*Tadarida mexicana*), 42 cave myotis (*Myotis velifer*), and 7 pipistrels (*Pipistrellus subflavus*). These three species are insectivorous and colonial. Two isolations were made in this survey. The first was from a pool of brain tissue from one *T. mexicana* and one *M. velifer*, while the second was from a torpid Mexican freetail bat collected on a wall near the ground. Both Mexican freetails had typical Negri bodies in their brains, but the cave myotis did not. The incubation period in mice was seven or eight days, and in the brains of the first and second passage mice, Negri bodies were demonstrated. Isolations were repeated a second time from the tissues of these bats and the identity of the virus was proven by serum neutralization.

Since this first survey, the Texas State Department of Health has continued its investigation of bat rabies and has, with regularity, made many more isolations from *T. mexicana*. In addition, Irons (66) reported isolation from another species, *Lasiurus borealis*, caught in Texas. This is a free-living, insectivorous, red bat, a species that migrates into Mexico. Recently, Grimes, Eads & Irons (67) described the isolation of the rabies virus from yet another species of colonial, insectivorous bat, *Chilonycteris personata*, collected in a cave near Vera Cruz, Mexico, and sent to the San Antonio Zoo.

Other isolations of the rabies virus from bats have been made in Texas,

by the Fourth Army Medical Laboratory at Fort Sam Houston. Burns & Farinacci (68) reported that between the first collection, on February 5, 1954, and the second trapping, on May 4, 1954, there was noted a malady among the bat population on the post. The affected bats exhibited various stages of paresis, muscular tremors, incontinence of urine and deranged behavior. The cause was believed to be the absorption of DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane) in the insecticides being intensively applied at that time. However, from a Mexican freetail bat caught prior to the insecticide spraying, the rabies virus was isolated. This bat was found hanging on a screen door and suffering from a spastic type of paralysis. The virus was also isolated from another freetail exhibiting signs of encephalitis when caught on May 6, 1954. Examination of the brains of these bats revealed the presence of Negri bodies in some of the Purkinje cells and similar inclusions in the cells of Ammon's horn. Intracerebral inoculation of mice resulted in the appearance of encephalitic symptoms in six or seven days. Negri bodies were found in the brain cells of the passage mice, and the identity of the virus was confirmed by serum neutralization. An additional 200 *T. mexicana*, that exhibited no abnormal behavior, were collected and bled. This blood was composed into 35 pools, heat-inactivated at 56°C. for 30 minutes, and used for neutralization studies in mice. Of the 35 pools, 65 per cent were found to neutralize 100 MLD₅₀ of virus fixe per 0.015 ml. sera.

In attempts to clarify the role that bats might play in the epizootiology of rabies, further studies were undertaken by this group during 1954 (69). Nine isolations were made from 335 Mexican freetail bats collected during a second survey. In five of the nine positive bat brains Negri bodies were found, while in the other four they could not be demonstrated. Inoculation of mice intracranially with suspensions of each of these positive brains induced signs of encephalitis, after an incubation period of from four to eight days. Histological examination of the brains of mice infected with one of these bat viruses revealed typical Negri bodies, with focal areas characterized by destruction of neurons, satellitosis, and moderate neuronophagia. There was no evidence of infection except in areas around neurons. Perivascular infiltration and glial nodules were not observed. Proof that the isolates were the rabies virus was accomplished by serum neutralization.

Burns, Farinacci & Murnane (69) described another serological survey in which 283 *T. mexicana* collected from counties in central, southern and western Texas were bled. The serum was divided into 44 pools, with each pool containing only the sera of bats caught in one area. Of the 44 pools 27 (61 per cent) neutralized 100 MLD₅₀, and eight (30 per cent) neutralized 1000 MLD₅₀ of virus fixe per 0.015 ml. sera. The number of positive pools from different areas varied from 16.6 to 70.0 per cent. These findings were offered as evidence of the survival of this species of bat after exposure to the rabies virus. In further studies, many more isolations of the rabies virus have been made from *T. mexicana* by this group. They also reported an

isolation from the small cave bat, *M. velifer*, trapped in Texas (70).

California.—In the summer of 1954, during the months of July, August, and September, a survey of bats in an attempt to isolate the rabies virus was undertaken by Enright *et al.* (71) at the School of Veterinary Medicine, University of California at Davis. In this study 211 bats of 14 different species were collected from an area of California in which rabies in wildlife and in bovine species is endemic. A suspension composed of the brain and submaxillary glands of each bat was inoculated intracranially into mice. From one of 17 Mexican freetails collected on July 20, 1954, the rabies virus was isolated. This bat showed no evidence of fighting, there were no scars visible, the fur was smooth, and the state of nutrition excellent. Histological section of the brain revealed no Negri bodies, no neuronal damage and no inflammatory reaction. Proof of the identity of the virus was obtained by serum neutralization tests. This isolation was confirmed by the Viral and Rickettsial Disease Laboratory, California State Department of Public Health. Some mice inoculated intracranially with this virus, after an incubation period of six to eight days, exhibited hyperactivity of the explosive type and convulsions that were sometimes terminated by death. Other mice developed a rapidly progressing paralysis which extended to the whole body at the time of death. Histological examination of the brains of these mice revealed Negri bodies that were well-formed and characteristic, and an encephalitic reaction.

Intracranial inoculation of other bats of the same species, and of another species (*Antrozous pallidus*), with tissues from the positive bat resulted in rabies in both species after an incubation period of 15 to 16 days. The Mexican freetails exhibited a furious and aggressive type of the disease, while the pallid bats more often showed paralysis of a progressive type. The aggressiveness of the inoculated freetails was demonstrated by their vicious fighting, that would result in bleeding wounds and loss of skin and teeth. The virus of rabies was isolated from the saliva and the brain of the first and second passage bats of both species. Histological examination of the brain of the passage bats revealed typical Negri bodies and encephalitis, while sections of the gasserian ganglion and the submaxillary gland were normal.

Inoculation of a gray fox in the masseter and gastrocnemius muscles, with brain tissue from a rabid bat, induced rabies after an incubation period of 18 days. This disease was characterized by an early onset of paralysis that started in the hindquarters and ascended rapidly. The disease terminated in complete paralysis after three days from onset. The virus was isolated from the brain of the fox and identified by neutralization with known antiserum. Histological examination of the fox brain did not reveal Negri bodies, but hyperemia, satellitosis and a mild focal gliosis were observed.

Two isolations were made in 1955 at the Viral and Rickettsial Disease Laboratory, California State Department of Public Health. The first was in the late spring of 1955, when Johnson (72) isolated the rabies virus from the salivary glands of a little California bat, *Moytis californicus*, found dead in a

fish pond. This bat is colonial and insectivorous, but not much is known of its migratory habits, although it is found in abundance in Mexico. The second isolation was made in October of 1955 by Lennette (73), from the brain of a bat that had bitten a man on the hand. The bat was not definitely identified, but was thought to be a freetail. The brain of this bat contained atypical inclusions, and the rabies virus was isolated from both the brain and salivary glands.

Rabies has been known in California for many years. It has been reported in humans and in many species of animals, both domestic and wild. Periodically, the incidence in sylvan rabies has been observed to increase, roughly following a seven year cycle. In 1954 and 1955, an increase in the number of proven cases of rabies in bovines, foxes and skunks was reported.

Montana.—On August 20, 1954, a bat was found in a flower garden in Hamilton, Montana. It was captured and submitted to the Rocky Mountain Laboratory of the Public Health Service, and was identified as the big brown bat *Eptesicus fuscus*. This is a colonial, insectivorous bat that is not known to migrate, but hibernates in caves during the colder months. Its range extends over the western part of the United States and into Mexico. The rabies virus was isolated from the brain of this bat by Bell, Hadlow & Jellison (74). Histological examination revealed typical Negri bodies in the pyramidal cells of the hippocampus, in the cerebral cortex and in the Purkinje cells. Frequently several of these inclusions could be observed in an individual neuron. Inflammatory changes were not observed with the exception of a minimal lymphocytic infiltration around some meningeal vessels, and a moderate hyperemia. Inoculation of mice intracranially with a 10 per cent suspension of the bat brain resulted in the death of two mice on the sixteenth day after inoculation. Subsequent serial passages, by the same route in mice, stabilized the incubation period to between 7 and 11 days. The brains of the first and second passage mice contained typical Negri bodies. Identity of the virus was proven by serum neutralization and cross-protection tests. Rabbits were inoculated intrathecally by cisternal puncture with a brain suspension from the second mouse passage. All except one contracted paralytic rabies after incubation periods of 14 to 30 days, and it was apparently normal after 60 days. Two bats were injected intracerebrally, one with virus fixe and the other with the bat strain of the virus from the second mouse passage. The bat inoculated with virus fixe exhibited only mild irritation when it was sacrificed. The other bat, inoculated with the bat strain of virus, became ill on the 14th day with a disease characterized by weak, tremulous, ineffective movements, and was sacrificed ten days later. The brain of each bat contained Negri bodies and the rabies virus was isolated from both.

Since rabies had not been reported in Montana since 1952, the source of infection for the bat was unknown. However, a canvas-covered trailer, recently arrived from Ohio, was parked in the neighborhood from which the bat was captured.

Ohio.—In August of 1955, the rabies virus was isolated from a suspension of the brains from three big brown bats (*Eptesicus fuscus*), collected in Columbus, Ohio (75). Studies of this virus are not yet complete.

There are other reports of isolations of the rabies virus from insectivorous bats in the United States, and of other attacks by some of these rabid bats on humans, but the reviewer has not been able to confirm these. Recent morbidity and mortality data for the United States lists 93 virus isolations from rabid insectivorous bats involving twelve species collected in eight widely-separated States of the Union (76).

CONCLUDING REMARKS

The rabies virus is well established in the bat population of the Americas, and is widely distributed, both geographically and among the various species of the order Chiroptera. Serological surveys and experimental observations indicate that hemophagous, fructivorous and insectivorous species may exhibit symptoms of the disease, either in the paralytic or furious form, or the infection may be entirely inapparent.

It has further been demonstrated that vampires and fructivorous bats are able to transmit the virus while they are suffering from the disease, and also as recovered or symptomless carriers. The insectivorous bats have the virulent virus in their saliva, but natural transmission has not been reported. The opportunity for transmission of the virus to other species of animals is enhanced by the feeding habits of the *Desmodontidae*, and this probably accounts for their prominent role in the rabies problem of Latin America. However, another circumstance which favors the opportunity for transmission of the rabies virus from bats to other animals is the constant search for food by the predator. There are reports showing evidence that the bats of the United States have been eaten by coyotes (77), snakes (78, 79), owls (80), skunks and opossums (81), mink (82) and raccoons (79). Therefore, the opportunity for transmission of the rabies virus from insectivorous bats to various animals can be appreciable. The rabies virus may be introduced into a bat colony by the escape of a bat from a rabid predator whose attention was distracted.

The remarkably constant characteristic of a spreading type of paralysis, often the first manifestation observed, is the primary feature of bat transmitted rabies in all species of animals, except in other Chiroptera. Paralysis coupled with the irregular negrigenesis is not without its parallel with other strains of the rabies virus. For example, the phenomenon called fixation, induced by serial intracranial passage in the same species of host, results in the development of a similar picture. The diminished virulence, for certain species of animals, has its counterpart in the attenuation of the Flury strain of the virus for mammals after many passages in the developing chick embryo. It is interesting to speculate whether or not these relationships indicate that the rabies virus has existed among the Chiroptera for a long

time, until a certain mutual tolerance in the host-virus relationship has become established.

Another unusual feature of the epizootics of paralytic rabies transmitted by bats has been the lack of correlation with canine rabies. In Trinidad, Mexico, and Brazil, it is significant that the early diagnoses of rabies in the bovine were seriously doubted, and even ridiculed, because dogs were not involved. From this observation it becomes important that the possibility of rabies be entertained in all unexplained outbreaks of disease characterized by an ascending paralysis.

Control of the cattle disease in Latin America has been successfully accomplished by preventive immunization. In Trinidad, humans have been protected from the disease after exposure by anti-rabies immunization. In the United States, some species of insectivorous bats inhabit buildings in tremendous numbers, in close contact with humans and animals. In these situations, elimination of bats by destruction and bat-proofing is feasible, and is practiced for esthetic reasons.

While the intimacy of habitat between bats, humans and animals affords opportunity for easy transmission of pathogens, the role of the insectivorous bat in the disease problems of the United States awaits clarification.

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THE PARASEXUAL CYCLE IN FUNGI¹

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INTRODUCTION

The present article is an essay rather than a review. The discovery in 1952 by Pontecorvo & Roper (1) of parasexual recombination in *Aspergillus nidulans*, i.e., recombination of hereditary determinants outside the sexual cycle, has been followed by research on two questions. First, which processes underlie what we now know to be a cyclical series of events, the parasexual cycle? Second, how widespread is the parasexual cycle among filamentous fungi, and what part does it play in their genetic systems, particularly in the case of species in which the sexual cycle is not known to occur? There is a corollary to the second question, relevant to the applied fields of phytopathology and industrial fermentations: what is the bearing of the parasexual cycle on variation in nature and in the factory? In nature because it might, and indeed Buxton's work (2) suggests that it does play a part in the variation of host-pathogen relationships; and in the factory because it might, and the work of Caglioti & Sermoniti (3) shows that it can be harnessed to the "breeding" of more productive strains.

With the exception of work on heterokaryosis, which we now know to be one of the steps in the cycle, practically all the published work on the parasexual cycle has been done either in our Laboratory or by workers previously associated with it, at the Istituto Superiore di Sanità, Rome, and at the Plant Pathology Department, Rothamsted Experimental Station.

Research on the first question has led to an understanding of the sequence of steps in the cycle, and to a working knowledge of some of these steps (4, 5). This working knowledge suggests new ways of investigation of the phenomenon observed by Buller (6), Quintanilha (7), Papazian (8), and Raper (9) in the *Basidiomycetes*, i.e., the formation of nuclei of new types in dikaryons. Furthermore, this knowledge is already sound enough for use in the mapping of chromosomes. In *A. nidulans*, for instance (5), certain operations for mapping are more conclusive and less laborious if the analysis is through the parasexual cycle than if it is carried out in the usual ways through the sexual cycle.

Research on the second question has ascertained that the parasexual cycle occurs in the asexual species *Aspergillus niger* (10, 11), *Penicillium chrysogenum* (3, 12) and *Fusarium oxysporum* (2), besides the homothallic *A. nidulans* in which it was first found. Recombination also occurs in *Streptomyces coelicolor* (13), but here it is not yet known whether or not the underlying process is the same as in the four species of fungi mentioned above. In short, a search for the parasexual cycle in four species of filamentous fungi

¹ The survey of literature pertaining to this review was completed in January, 1956.

has found it in all of them: it is legitimate to conclude that it cannot be a rare oddity.

As to the part which the parasexual cycle plays in nature in the systems of heredity and variation of the species in which it occurs, there is only the remarkable work on *F. oxysporum* to quote (2). Variation in natural populations is a huge field of research in which the mycologist, including the industrial mycologist, and the phytopathologist should have a more direct interest than the geneticist. Yet at a discussion at the New York Academy of Sciences as recently as 1954, of fourteen papers on the topic "Speciation and Variation in asexual fungi" (14), only one realised what a change in outlook is imposed by the existence of the parasexual cycle.

THE ELEMENTS OF THE PARASEXUAL CYCLE

The parasexual cycle is known in great detail in *A. nidulans* (15), where the existence of a sexual cycle permits a thorough control of each step by means of standard genetic techniques. In the other species mentioned before, the complete cycle has been identified in *P. chrysogenum* (3, 12, 15, 16, 17). In *A. niger* and *F. oxysporum* some of the steps are not yet quite established.

The steps of the cycle are the following: (a) Fusion of two unlike haploid nuclei in a heterokaryon. (b) Multiplication of the resulting diploid heterozygous nucleus side by side with the parent haploid nuclei in a heterokaryotic condition. (c) Eventual sorting out of a homokaryotic diploid mycelium which may become established as a strain. (d) Mitotic crossing over occurring during multiplication of the diploid nuclei. (e) Vegetative haploidisation of the diploid nuclei.

Events (a), (d) and (e) occur at rather low rates. For the sake of orienting the reader, let us say: 1 in 10^6 or 10^7 for fusion of two unlike haploid nuclei in a heterokaryon, 1 in 500 for the incidence of crossing over per diploid nucleus and 1 in 1000 for the haploidisation of diploid nuclei. The actual variation in these figures from one combination of strains to another and from one species to another is very large. However, they reveal one of the reasons why diploid strains have not been identified in nature and why they are unlikely to be identified without a deliberate selective search: in the absence of selection against haploids these should outnumber the diploids as the ratio of the rate of haploidisation versus that of fusion, i.e., 1000 to 1.

Fusion of unlike nuclei.—This process is inferred from the fact that, in a heterokaryon, diploid nuclei arise which are heterozygous for all the markers in which the two types of haploid nuclei present in the heterokaryon differed. In fact the original technique by Roper (18) for the synthesis, identification, and isolation of diploids was precisely based on the expectation, on general grounds, that a mycelium heterozygous for known markers should show certain properties. These properties are expected to be different, in predictable ways, from those of the two mycelia carrying either of the two kinds of haploid nucleus or from those of the heterokaryon itself. In the laboratory it is only a matter of choosing, for the formation of a heterokaryon, a suitable

combination of strains differing in known nuclear markers. For instance, if the two strains, by virtue of two different mutant presumably recessive alleles, require two different growth factors, the heterokaryon and the diploid heterozygote derived from it will require neither, but the heterokaryon and the heterozygote will be distinguishable in one important respect. The heterokaryon, carrying nuclei of two kinds, will give origin to a proportion of hyphae homokaryotic of either kind. There is more: in a species with uninucleate conidia the two kinds of nuclei will be invariably sorted out in the formation of conidia. In the heterozygote, on the other hand, neither type of segregation will occur, except for the rare mitotic segregation and recombination to be described later. The conidia from the heterokaryon will not grow on nonsupplemented medium, but if among them there is an odd one carrying a diploid heterozygous nucleus, this one will be able to form a colony. Under such selective conditions, the diploid may also become established as a sector out of a heterokaryotic colony: this is of common occurrence with *A. niger* (11) and *P. chrysogenum* (12).

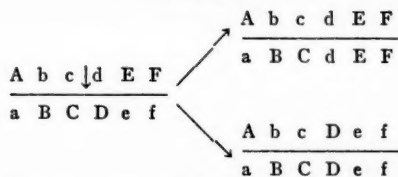
The heterozygous diploids, besides having the phenotypic properties expected on general grounds and segregating mitotically, as described below, have a volume and deoxyribonucleic acid content of uninucleate conidia double that of haploid conidia (4, 18, 19).

Nothing is known of the way in which fused nuclei arise. We only know that they do arise and crude measurements of the rate at which they arise are available in some cases (15). It would be interesting to know something about this process, which may well be only a rare accident, but this knowledge is not essential to an understanding of the parasexual cycle, just as a knowledge of the details of fertilisation is not essential for understanding the biological consequences of sexual reproduction.

Recently Elliott (20) has synthesised triploid strains of *A. nidulans* by applying Roper's technique to heterokaryons between haploids and diploids. These diploids must, of course, be homozygous for one or more recessive markers, suitable for selection.

Mitotic crossing over.—During multiplication, heterozygous diploid nuclei give origin occasionally to nuclei still diploid but homozygous for one or more of the markers previously heterozygous. Early in the work (1, 10, 11, 18, 21) it became clear that similarly to what Stern (22) found in *Drosophila*, mitotic crossing over could account for this segregation. This became certain after investigating diploids of *A. nidulans* heterozygous for linked markers, the locations of which along one chromosome were known (4). Since then work with a large number of different diploids has amply confirmed this conclusion. The matter has also been clinched by the recovery of the two complementary products of mitotic crossing over in single nuclei (23, 24).

Mitotic crossing over produces from, say, one out of 500 heterozygous nuclei A/a, two daughter nuclei A/A and a/a. If the nuclei are heterozygous for a number of linked markers, segregation in any one nucleus occurs only for the markers distal to the position of exchange in that nucleus, e.g.:



The proximal markers (i.e., those between the centromere and the position of exchange) remain unaffected, and so do those on the other arm of the same chromosome pair and those on different chromosome pairs.

The incidence of exchanges is sufficiently rare to permit us to disregard the occurrence of more than one exchange in any one nucleus. This means that the order of genes along the chromosomes can be determined easily by means of mitotic crossing over, and even more unequivocally than by means of meiotic crossing over (5).

The positions of the centromeres can also be determined. In the example just given, if we assume that the centromere is between the loci c/C and d/D , then homozygosity at the d/D locus would always go with homozygosity at e/E and f/F but not at a/A , b/B or c/C , and homozygosity at c/C would always go with homozygosity at b/B or a/A but not at d/D , e/E or f/F .

In *A. nidulans* mapping of two chromosomes by means of mitotic crossing over has already led to the building of "mitotic" maps with a total of 12 loci and two centromeres (5). These maps confirm absolutely the linear order of loci and centromeres determined independently by means of meiotic analysis via the sexual cycle.

In view of the relative rarity of mitotic crossing over, an essential point for mitotic mapping is to have available a number of markers suitable for selection. For example these can be "visible" markers, such as differences in colour of the conidia detectable even in a small number of heads. Or they may be markers conferring to the mycelium homokaryotic for one allele properties which lend themselves to selection: for instance, homozygotes for an allele conferring resistance to Acriflavine (25) are much more resistant than heterozygotes; hence the homozygotes arising by mitotic crossing over from heterozygotes can be selected as vigorous sectors out of heterozygous colonies grown in the presence of Acriflavine.

Another type of useful markers are "suppressors," i.e., recessive alleles restoring the ability to grow in the absence of a particular growth factor to strains homozygous for mutants determining requirement for that growth factor. In this case a diploid heterozygous for the "suppressor" and homozygous for the allele determining the requirement requires the growth factor, but a homozygote for the suppressor, produced by mitotic crossing over does not, and can therefore be selected on a medium lacking the growth factor (5, 24).

In the genetics of phytopathogenic fungi, genes affecting the host range can conceivably be used as selective markers.

The absolute incidence per nucleus of mitotic crossing over is 500 to 1000 times lower than that of meiotic crossing over. The question then arises of whether or not the relative distribution along any one chromosome is the same for mitotic and meiotic crossing over. A first answer to this question is available (5): the relative distribution along two chromosomes of *A. nidulans* with a total of nine intervals studied is definitely different at meiosis as compared with mitosis. The work so far does not reveal any general feature in this difference. In two arms there is a relative excess of mitotic crossing over in an interval of about 20 units next to the centromere but in a third arm a gross excess is in an interval of 10 units starting 20 units away from the centromere.

It has been suggested (5, 26) that analysis via mitotic crossing over in tissue cultures might be used for mapping the chromosomes of slow breeding higher organisms, including man.

All the detailed work on the process of mitotic crossing over has been done with *A. nidulans* because in this species it is possible to check any deduction by means of ordinary genetic analysis. In species in which a sexual cycle is unknown this cross check is impossible. For this reason the preliminary work with *A. niger* (11) and *P. chrysogenum* (12) did not even attempt to use mitotic crossing over for identifying linkage groups and constructing chromosome maps.

The knowledge gained in *A. nidulans* by the analysis in parallel via mitotic and via meiotic crossing over has now made it possible to start this kind of work with completely asexual species. The first linkage group in an asexual species, *P. chrysogenum*, has been identified by Sermoniti (27).

Haploidisation.—Segregation of the markers for which a diploid is heterozygous occurs during vegetative multiplication not only as a consequence of mitotic crossing over. It is also the consequence of a process of haploidisation in which whole chromosomes, but not chromosome parts, re-assort at random (4).

The details of the process of haploidisation are almost completely unknown. In *A. nidulans* diploid nuclei produce haploids at a rate lower than that at which they undergo mitotic crossing over. For instance, diploids homozygous for the most distal marker in one chromosome arise anything from 1.5 to 10 times as frequently as haploids carrying the marked chromosome.

A working hypothesis used for planning further work was that haploidisation is the consequence of accidental breakdown at mitosis in the separation of the chromatids to the two poles: in a proportion of cases one daughter nucleus arises with a single set of chromosomes (4). Breakdowns of this kind are well known in higher organisms.

Two of the testable consequences of this hypothesis are that the same process which produces haploid nuclei should also: (a) produce diploid nuclei homozygous for whole chromosomes, and (b) produce aneuploid nuclei. Both predictions have been verified in unpublished work of Pontecorvo &

Käfer on *A. nidulans*. The quantitative aspects, however, are such that these results do not constitute as good evidence as they would seem in support of the working hypothesis. Briefly, it is found that diploids homozygous for a whole chromosome, well marked on both arms, are homozygous for other chromosomes less frequently than expected from random distribution.

On the other hand the use of diploids marked on several chromosomes has shown that the majority of haploids originate as aneuploids, i.e., they are "monosomic" for some chromosomes but still "disomic" for others. This imbalance puts a strong premium on nuclei which eliminate the extra chromosomes, so that the aneuploid repeatedly throws out more vigorous sectors which remain constant thereafter. Analysis of these sectors shows that in a proportion of cases the disomic chromosome pair was still heterozygous. The situation is in most respects the same as that of the *Neurospora* disomics (28).

It is clear that a considerable proportion of the products of haploidisation must be imbalanced and nonfunctional and therefore be eliminated. The process of haploidisation is at present under study, both by means of diploid strains marked on both members of each chromosome pair and by means of triploids.

SIGNIFICANCE OF THE PARASEXUAL CYCLE

The use of markers has permitted to follow through heterokaryosis, diploid heterozygosis, and haploidisation the fate of various parts of the genotype. The result of the parasexual cycle is that starting from a culture containing a mixture of genetically different haploid strains, one would end up ultimately with a much more varied mixture. The latter would include: (a) haploid strains like the starting ones; (b) haploid strains which have recombined in all possible ways the chromosomes and chromosome parts of the starting strains, and (c) a small proportion of diploid strains homozygous and heterozygous for all possible associations and recombinations of markers of (a) and (b). It is not to be excluded that also a small proportion of triploids, and higher polyploids, would be present. The actual proportions of the various strains would be determined in any one case by the external conditions which would keep the less fit combinations at low levels.

Clearly a genetic system based on the parasexual cycle has all the elements which are found in a genetic system based on sexual reproduction, and some novel ones. Within a population, it provides for the storage, both in heterokaryotic and heterozygotic condition, of a large amount of gene diversities originated by mutation or by any other mechanism which may produce such diversities. It also provides for the re-assortment of all these diversities both in haploid and in diploid condition, ready for the sieve of natural (or artificial) selection.

The main novel features of the parasexual cycle are two. One is that though it is made up of steps which, considered together, are more or less like those of the sexual cycle, the precise sequence of these steps is not fixed as in

the sexual cycle. Moreover, haploidisation is achieved rather wastefully, though it occurs only in a very minor proportion of all diploid nuclei. The second feature is the occurrence, even in one mycelium, of both heterokaryosis and heterozygosis. Heterokaryosis may involve nuclei of the same ploidy or of different ploidies, and even more than two types of nucleus in one mycelium. This joint play of heterozygosis and heterokaryosis permits a latitude in the storing of gene variation vastly greater than in other organisms. For instance, in most higher organisms gene variation can only be stored in diploid heterozygotes, and in the *Basidiomycetes*, as far as it is known, only in heterokaryons or dikaryons. On the whole, the parasexual cycle seems to be less perfect but more flexible than the sexual cycle.

There is no need to make a case for harnessing such a system in the artificial selection of industrially valuable moulds. Obviously in an asexual species the parasexual cycle can be used for "breeding" improved strains just as the sexual cycle is used for producing better varieties of flowering plants, and, for example, better breeds of dogs and sheep. Technically the difficulties are far smaller in industrial microbiology than in horticulture or animal breeding.

It is legitimate, however, to ask whether the parasexual cycle plays more than an irrelevant part in natural populations. The answer is that we do not know yet, but it would be surprising if a system with potentialities as great as those of sexual reproduction were merely a laboratory curiosity.

Admittedly, variation in the characters used for the laboratory work (growth factor requirements, color of conidia, etc.) has not been so far of the kind which makes up most of the differences between populations in nature. Also admittedly, the very little evidence available as to heterokaryosis in nature shows that while a high proportion of isolates from nature are heterokaryotic, monokaryotic strains collected in different localities more often than not refuse to form heterokaryons with one another (29).

Fortunately variation of a type with the greatest adaptive significance has now been added to the list by the work of Buxton (2). Two races of the pathogen *Fusarium oxysporum pisi* differ in host range: one is pathogenic for the pea variety "Onward" but not for the variety "Alaska," and the other for both. Neither race is pathogenic for "Delwiche Commando." A diploid synthesised by means of Roper's technique from the two races had the broader host range (both "Onward" and "Alaska") and of the recombinants from this diploid three were also pathogenic for "Delwiche Commando": more precisely, two were pathogenic for "Onward" and "Delwiche" though they differed in nutritional requirements, and one for all three, "Onward," "Alaska," and "Delwiche."

The fact that parasexual recombination can determine variation in the host range of pathogens opens a wide field of research both fundamental and applied mainly to phytopathology, i.e., the study of what part recombination plays in the origin of "new" pathogenic races. This question, asked rather academically in 1947 (30), becomes now quite practical.

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